

A Study of Excitation- Contraction Coupling in Skeletal Muscle Fibres

With Special Reference to Drug Action

A. Rauf Khan

Lund 1981



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A Study of Expiration-
Contraction Coupling in
Skeletal Muscle Fibers
With Special Reference to Type IIb

A. Raut Kinn



In the name of Allah the Gracious, the Merciful

O, my Allah, I am grateful to You from the depths of my heart. Everything, I have, is because You have been Gracious and Merciful to me. My intellect, abilities and capabilities are all due to Your Grace. You gave me the health and opportunities to work, and I have no words to thank You for all Your favours. Through Your beloved prophet Muhammad, on whom be peace, You guided me to the untouchable truth,

"the universe has been subjected by law
for the service of humanity (Quran 31:21)"

without which no scientific progress would have been possible.
Through Your beloved Messiah You told me that

"in each and every cell/particle Allah has
laid innumerable properties and secrets so that
no one can fully encompass the knowledge of these
profound mysteries (Durre-Samin)"

which has been a source of immense encouragement for me.

O, my Lord, I supplicate to You that looking into the glory of Your creation may prove to be a source of a living faith in You and a source of establishing a good contact with You, and that I may always use my knowledge for the benefit of humanity.

The dissertation is based on the following papers which in the text will be referred to by their Roman numerals:

- I. Khan, A.R. & Edman, K.A.P. 1979. Effects of 4-aminopyridine on the excitation-contraction coupling in frog and rat skeletal muscle. *Acta Physiol Scand.* 105: 443-452.
- II. Wollmer, P., Wohlfart, B. & Khan, A.R. 1981. Effects of 4-aminopyridine on contractile response and action potential of rabbit papillary muscle. *Acta Physiol Scand.*, (in press).
- III. Khan, A.R. 1979. Effects of diethyl-stilboestrol on single fibres of frog skeletal muscle. *Acta Physiol Scand.* 106: 69-73.
- IV. Khan, A.R. & Wohlfart, B. 1981. Effects of diethyl-stilboestrol on contractile response and action potential of rabbit papillary muscle. *Acta Physiol Scand.* 111: 201-204.
- V. Khan, A.R. 1980. Mechanism of action of pentobarbital on the contractile system of isolated frog muscle fibres. *Acta Physiol Scand.* 108: 405-409.
- VI. Khan, A.R. 1981. Influence of ethanol and acetaldehyde on electro-mechanical coupling of skeletal muscle fibres. *Acta Physiol Scand.* 111: 425-430.

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ABBREVIATIONS

4-AP	4-aminopyridine
DES	Diethyl-stilboestrol
SR	Sarcoplasmic reticulum
(E-C) coupling	Excitation-contraction coupling

INTRODUCTION

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The importance of calcium in muscle contraction was recognized by Heilbrunn and Wiercinski (1947) who, by injecting various ions into single muscle fibres showed that calcium was the only cation with physiological action that caused muscle fibres to contract. The initial step in excitation-contraction (E-C) coupling is membrane depolarization which conveys a message into the interior of the muscle fibre through the transverse tubule-system (for review see Sandow 1965; Endo 1977). In response to this signal calcium is released from the storage sites, probably the terminal cisternae of the sarcoplasmic reticulum (SR), into the myoplasm. The calcium ions activate the myofibrils and the fibre contracts (for review see Sandow 1965; 1970; Ebashi 1976; Endo 1977). Relaxation requires the removal of calcium from the myoplasm. In skeletal muscle the presence of an extractable factor playing an important role in relaxation was suggested by Marsh (1952) and Bendall (1953). Subsequent work identified the relaxing factor ("Marsh and Bendall factor") as the sarcoplasmic reticulum (Nagai et al. 1960; Muscatello et al. 1962) which can sequester calcium by an energy dependent process (Hasselbach and Makinose 1961; Ebashi 1961; Ebashi and Lipmann 1962; Hasselbach 1964; Weber 1966; for review see Sandow 1965; Ebashi and Endo 1968).

Isolated SR membranes from skeletal muscle form closed vesicles which contain calcium stimulated ATPase and are capable of accumulating calcium from the surrounding medium (Hasselbach and Makinose 1961; Ebashi 1961; Ebashi and Lipmann 1962; Hasselbach and Makinose 1963; Hasselbach 1964; Weber 1966; for review see Tada et al. 1978). On the basis of this evidence it is now generally held that the SR in intact muscle is intimately involved in relaxation. Evidence in support of this idea is provided by the demonstration of Ebashi and Ebashi (1962) and Weber et al. (1963) that the

microsomal fraction of the SR removes bound calcium from various actomyosin systems and thereby causes relaxation.

It has been shown that removal of calcium from the extracellular medium depolarizes the muscle fibre and renders it inexcitable to electrical stimulation (Edman and Grieve 1961; 1964; Jenden and Reger 1963; Andersson and Edman 1974). Replacing calcium with lanthanum restores the resting membrane potential and thus the excitability of the muscle fibre and gives normal twitch response (Andersson and Edman 1974). This finding suggests that skeletal muscle does not immediately depend on extracellular calcium influx for its contractile activity (for review see Caputo 1978). Calcium is released and taken up again by the tubular system of SR, i.e. the muscle fibre behaves virtually as a closed system in this sense. Unlike the situation in skeletal muscle, cardiac and smooth muscles are critically dependent on extracellular calcium influx (Edman and Schild 1962; Casteels et al. 1977; Bengtsson 1978; for review see Fozzarard 1977). Drugs affecting the metabolism of calcium may, therefore, differ in their effects depending upon which calcium pool plays immediate role for activation in the particular muscle, the extracellular or the intracellular source of calcium.

Although depolarization of the muscle fibre membrane is the first physiological step for inducing muscle contraction, agents like caffeine are known to induce muscle contraction without depolarization of the cell membrane (Axelsson and Thesleff 1958; Weber and Herz 1968; Geffner et al. 1975; Lüttgau and Oetliker 1968). Extensive work has been done to elucidate the mechanism by which caffeine induces contractures and it has been suggested that caffeine enhances the calcium-induced calcium release from the intracellular stores (Endo 1975; for review see Ebashi 1976).

Many drugs affect the calcium uptake properties of isolated SR membranes (e.g. Balzer et al. 1968; Weber and Herz 1968; Balzer 1972; Van Winkle 1976). However such studies may not fully account for the pharmacological effects produced by the drug in a muscle cell, as the drug may not penetrate the cell membrane. Drugs may affect the release of calcium by affecting the action potential duration or by changing the mechanical threshold (Sandow et al. 1965; Edman et al. 1966). They may also penetrate the cell membrane and exert a more direct action on the release and reuptake mechanisms of calcium.

The present studies concern the effects of 4-aminopyridine (4-AP), diethyl-stilboestrol (DES), ethanol and acetaldehyde and pentobarbital on the excitation-contraction coupling in frog skeletal muscle fibre. Twitch and tetanus responses were recorded and the time course of the responses analyzed.

Contractures induced by different concentrations of caffeine has been used to study the mechanism of action of drugs which have a more direct effect on release and uptake of calcium in muscle fibres (Endo 1975). In the present studies, caffeine contractures were also produced in order to elucidate the mechanisms underlying the observed pharmacological effects.

Graded depolarization of the muscle fibre membrane by raising the extracellular potassium concentration was used to study the influence of drugs on the mechanical threshold, i.e. the depolarization level at which the mechanical response is initiated.

Resting membrane and action potentials were recorded and the time course of the action potential was related to the change in mechanical performance.

The present investigation also includes a study of the effects of diethyl-stilboestrol (DES) and 4-aminopyridine (4-AP) on the contractile response and action potential of papillary muscle from rabbit heart. The pharmaco-

logical effects observed in skeletal and cardiac muscle were correlated with functional differences in the calcium metabolism of the two types of muscle.

METHODS

1. Preparations and mounting.

- a. Single muscle fibres were prepared from the ventral head of the semi-tendinosus muscle of Rana temporaria. Measurements of resting and action membrane potentials were made on small muscle fibre bundles (containing 5-10 fibres). The fibres were mounted horizontally in a perspex chamber. The temperature was controlled by circulating a glycol-water mixture through jackets surrounding the chamber and the containers of the solutions. The fibre was mounted between a force transducer and an adjustable stainless steel hook. The chamber contained 1.2 ml of solution and allowed rapid exchange of the bathing solution.
- b. Lumbrical muscles dissected from the front legs of Sprague-Dawley rats were used in some experiments.
- c. Papillary muscles were dissected from the right ventricle of small rabbits. The preparation was mounted horizontally in a thermostatically controlled bath between a force transducer and a steel hook. The length of the muscle was adjusted to 95 % of the length for optimum force production.

2. Stimulation

- a. Frog muscle fibre or the rat Lumbrical muscle was stimulated by passing current through an assembly of platinum pair electrodes placed along the length of the fibre or the rat muscle. Pulses of 0.2 ms duration were used and the stimulation strength was adjusted to be supramaximal for each electrode pair.

- b. Rabbit papillary muscle was stimulated to contract isometrically by passing current between two platinum electrodes placed at either side of the muscle. The stimulus pulse was a unidirectional rectangular wave of 2 ms duration and with an amplitude about 50% above the threshold value. The muscle was paced to contract at a rate of 0.67 Hz.

3. Recording of mechanical and electrical responses.

- a. Tension was recorded by means of an RCA 5734 mechanoelectric transducer and in later experiments by means of a semiconductor strain-gauge transducer. Signals from the transducer were displayed either on a Tektronix 502A or on a Tektronix 5107N storage, oscilloscope and in some cases simultaneously recorded on paper by means of an Elema-Schönander ink-jet recorder.
- b. Conventional glass capillary electrodes filled with 2.5 M KCl and with a resistance of 10-30 MΩ were used to record the membrane potential. The action potentials were displayed on the above mentioned oscilloscope and photographed on 35 mm plus-X pan film or on orthochromatic film.

4. Film analysis.

Measurements of the oscilloscope records were made at 10 x magnification in a Nikon model 6 C profile projector.

- a. In the frog muscle fibre action potential duration was measured at -25 mV level.
- b. In cardiac muscle action potential duration was defined as the time from the stimulus to the repolarization of the action potential at a voltage of -40 mV.

Further experimental details of the apparatus, materials and techniques used are given in papers I-VI.

RESULTS

1. Effects of drugs on isometric twitch and tetanus responses of frog muscle fibres.

4-Aminopyridine (4-AP, 3.0 mM), diethyl-stilboestrol (DES, 5.0 μ M), acetaldehyde (1.8 mM) and pentobarbital (0.5 mM) greatly potentiated the isometric twitch response of the isolated frog muscle fibre. The magnitude of twitch potentiation was inversely related to the twitch/tetanus ratio recorded in control Ringer solution. An analysis of the time course of the twitch response showed that 4-AP, DES and acetaldehyde increased the time to peak twitch tension with no significant effect on initial rate of rise of tension. An increase in the initial rate of force development was, however, observed with pentobarbital. DES and pentobarbital increased the half time for relaxation by a factor of 5.5 and 3.6 respectively whereas this factor was only 1.7 for 4-AP and 1.1 for acetaldehyde.

The effects of ethanol on twitch response were tested in concentrations ranging between 0.01 and 0.5 M. In low concentrations (0.01-0.05 M), ethanol had no significant effect on the contractile activity of the isolated muscle fibre. However, with higher concentrations (0.4-0.5 M) the amplitude of the isometric twitch was greatly depressed by ethanol together with a decrease in the rate of force development. No potentiation of the twitch was observed with ethanol in any concentration studied.

4-AP (3.0 mM), DES (5.0 μ M), acetaldehyde (1.8 mM), pentobarbital (0.5 mM) and ethanol (0.4 M) had no significant effect on the amplitude of the isometric tetanus. However, drugs like DES and pentobarbital which caused a marked prolongation of the relaxation phase of the single twitch also greatly increased the time for half decay of the tetanic tension. The tetanic response was depressed by ethanol when its concentration was raised from 0.4 to 0.5 M.

2. Effects of 4-AP on isometric twitch and tetanus responses of mammalian muscle.

The effects of 4-AP were also studied on mammalian muscle using the curarized rat lumbrical muscle. In this preparation also 4-AP ($> 0.1 \text{ mM}$) potentiated the twitch response without significantly affecting the tetanic tension. There was an increase in the time to peak twitch tension but, in contrast to the finding in frog skeletal muscle, there was no significant increase in the time to half relaxation.

3. The antagonistic action of twitch potentiating agents on dantrolene depressed isometric twitch response of frog muscle fibre.

Dantrolene is a powerful muscle relaxant which greatly suppresses the twitch response of the muscle fibre. This action of dantrolene is most likely attributable to inhibition of calcium release in response to membrane excitation (see further Discussion). 4-AP (3.0 mM), DES ($5.0 \text{ }\mu\text{M}$), acetaldehyde (1.8 mM), in concentrations producing maximum twitch potentiation, effectively counteracted the twitch depressant effect of dantrolene ($9.0 \text{ }\mu\text{M}$). Pentobarbital was more potent in this respect because, at a submaximal twitch potentiating concentration (0.2 mM), it almost normalized the rate of rise and amplitude of the twitch response of a dantrolene treated muscle fibre.

4. Role of extracellular calcium in drug induced twitch potentiation.

Drugs may potentiate the twitch response of the isolated muscle fibre by changing the trans-membrane influx of calcium. To test this hypothesis, effects of 4-AP were studied on single muscle fibres immersed in calcium free Ringer solution. In such experiments calcium was replaced by lanthanum in low concentration (0.05 mM) to maintain the normal resting membrane potential and excitability of the muscle fibre. No significant difference in the twitch potentiating effect of 4-AP was observed with or without calcium in the extracellular medium. The implication of this finding is discussed in detail below.

5. Effects of drugs on caffeine induced contractures.

In skeletal muscle fibres caffeine (1.5-5.0 mM) induces contractures by initiating calcium release from the intracellular stores. The peak contracture tension plotted against log caffeine concentration forms an S-shaped curve. The drugs which markedly prolonged the relaxation of twitch and tetanus responses, i.e. DES (5.0 μ M) and pentobarbital (0.5 mM), shifted the caffeine concentration-tension curve towards lower caffeine concentrations. 4-AP (3.0 mM) and acetaldehyde (1.8 mM) had no significant effect on the caffeine response.

6. Effects of 4-AP on mechanical threshold.

The muscle fibre can be made to contract by increasing the extracellular potassium concentration. It has been shown by Hodgkin and Horowicz (1960) that the tension development in a single muscle fibre is related to log potassium concentration by a steep S-shaped curve. Such a relationship between graded depolarization and tension development provides a measurement of the mechanical threshold, i.e. the level of depolarization at which the contractile response is initiated. The results showed that 4-AP had no significant effect on the potassium concentration-tension relationship and therefore did not affect the mechanical threshold.

7. Effects of drugs on the electrical activity of the muscle fibre.

4-AP (3.0 mM), pentobarbital (0.5 mM), DES (5.0 μ M) and acetaldehyde (1.8 mM) caused no significant change in the resting membrane potential of the frog muscle fibre. No changes in the kinetics of the action potential were observed in the presence of DES and acetaldehyde. 4-AP and pentobarbital, on the other hand, markedly reduced the rate of repolarization of the action potential and thereby prolonged the total duration of the action potential.

In the presence of ethanol (0.4 M) there was a reduction in the resting membrane potential and in the maximum rate of rise of the action potential as observed in 4 out of 11 muscle fibres. These changes, however, were not statistically significant ($P > 0.05$). The overshoot and the maximum rate of repolarization of the action potential were significantly decreased by ethanol and the total duration of the action potential was prolonged.

8. Effects of drugs on the contractile response and action potential of rabbit papillary muscles.

The effects of DES (1.0-10.0 μM) were studied on isometrically contracting isolated rabbit papillary muscles. Exposure to 1.0 μM DES did not significantly affect force production. DES in 10.0 μM concentration, however, reduced both maximum rate of force production and peak isometric force to nearly half their control values. There was no significant change in the time to peak force and in the time from peak force to half relaxation. The duration of the action potential was shortened in 4 out of 6 muscle preparations but this effect was not statistically significant.

The effects of 4-AP were studied on papillary muscles isolated from reserpinized rabbit hearts. In concentrations ranging between 50 and 800 μM , 4-AP increased the peak force by 20-60% of the control value. The increase in maximum amplitude of contraction was accompanied by an increase in the rate of force production and an increase in time to peak tension. Within the concentration range studied (50-800 μM), 4-AP prolonged the total duration of the action potential by 20-70% of the control value. The prolongation of the action potential was mainly attributable to a marked decrease in the rate of repolarization of the action potential.

DISCUSSION

There is evidence that drugs may affect contractility in skeletal muscle either by exerting an effect on the plasma membrane and thereby changing the kinetics of the action potential or by influencing the function of intracellular organelles involved in the metabolism of activator calcium. The effects of 4-AP on the kinetics of the action potential and the implication of this effect on the mechanical response are first considered. 4-AP has little effect on the rate of depolarization but reduces the rate of repolarization of the action potential markedly (paper I). Repolarization involves an outward current of potassium across the sarcolemma and the slowing of the repolarization by 4-AP can, at least in skeletal muscle fibres (Gillespie and Hutter 1975; Molg6 et al. 1975), be attributed to reduced potassium conductance during membrane excitation. As a result 4-AP prolongs the total duration of the action potential. A similar action of 4-AP has been suggested to be involved in the prolongation of action potential duration in the nerve terminal (Pelhate and Pichon 1974; Molg6 et al. 1977; Lundh and Thesleff 1977; Kirpekar et al. 1977; Illes and Thesleff 1978; Lundh 1978). The same mechanism may indeed also apply for cardiac muscle, for recent investigations on rabbit papillary muscle (paper II) have demonstrated that 4-AP prolongs the action potential duration of the cardiac cell by selectively reducing the rate of repolarization (paper II, and Yanagisawa and Taira 1979).

The effects on the action potential may, to a great extent, account for the enhanced twitch response by 4-AP. It is now generally believed that in skeletal muscle the action potential duration governs the time during which activator calcium is released from the intracellular stores into the myofibrillar space (e.g. Sandow 1965; Sandow and Preiser 1964; Sandow et al. 1965; Edman 1965; Edman et al. 1966; Taylor et al. 1972).

On this basis 4-AP, by prolonging the action potential duration, would increase the release of activator calcium resulting in a higher peak calcium concentration at the contractile sites. This in turn would require a longer time for the elimination of calcium from the contractile machinery. As the peak concentration of the calcium is likely to be sufficiently high to fully activate the contractile system, an increase in the release of activator calcium would merely cause a prolongation of the active state (Andersson and Edman 1974). This would result in a twitch response similar to that obtained in the presence of 4-AP, i.e. an increased peak amplitude and a later attainment of peak tension with no significant change in the initial rate of rise of tension. In line with the view that the contractile system reaches mechanical saturation during a tetanus (cf. above), 4-AP did not affect the total amplitude of the tetanic response (paper I).

The twitch potentiation by 4-AP in skeletal muscle is probably not due to increased trans-membrane calcium influx during the prolonged action potential, for the drug potentiated the twitch even when the muscle fibre was immersed in calcium free Ringer solution. Such studies could be carried out when calcium was replaced by lanthanum in a concentration sufficient to maintain normal resting potential and twitch response of the muscle fibre (Andersson and Edman 1974). All the evidence suggests that lanthanum does not penetrate the cell membrane (Laszlo et al. 1952; Lesseps 1967; Langer and Frank 1972; for review see dos Remedios 1981) and by itself causes only a slight potentiation of twitch in low concentration (paper I, and Andersson and Edman 1974). These results are consistent with the view that skeletal muscle, for its contractile activity, does not directly depend on extracellular calcium influx (Armstrong et al. 1972; Andersson and Edman 1974; for review see Ebashi 1976; Endo 1977; Caputo 1978).

4-AP also affects the kinetics of the action potential in cardiac muscle. The enhanced twitch response of the isolated papillary muscle is associated with a marked prolongation of the action potential. The contractile response of cardiac muscle is, to a great extent, dependent on trans-membrane calcium influx (Fozzard 1977; Fabiato and Fabiato 1979) during excitation. An increased calcium uptake, by the ventricular cell, during the prolonged action potential is therefore suggested to be responsible for the positive inotropic effect of 4-AP in cardiac muscle (paper II). Furthermore, the prolonged action potential will cause a greater release of activator calcium from the cellular store (increased duration of release). This will contribute to the force enhancement and explain the increase in the time to peak tension.

Diethyl-stilboestrol (DES) has no effect on the kinetics of the action potential that would account for the twitch potentiation produced by this drug. The amplitude of the twitch is enhanced by DES without any significant change in the initial rate of rise of tension. However, the rate of decay of twitch and tetanus tension is markedly reduced. It is, therefore, reasonable to believe that DES exerts an inhibitory action on the SR calcium pump, slowing the resequestration of activator calcium. In line with this idea DES strongly inhibits the ATP supported calcium uptake by SR isolated from rabbit skeletal muscle (Khan and Balzer unpublished data). It appears likely that inhibition of calcium reuptake by SR causes accumulation of activator calcium at the contractile sites during its continued release from the cellular stores. This would result in an enhanced twitch amplitude with a reduced rate of relaxation, i.e. an effect like that produced by DES. The above interpretation is in line with the idea that in skeletal muscle calcium releasing sites are different from the calcium uptake sites (Winegard 1965a; 1965b; 1968; for review see Caputo 1978) and, furthermore, that the calcium release and reuptake processes operate simultaneously.

DES also affects the contractile response of a single muscle fibre produced by caffeine (paper III). It is known that caffeine induces contractures (Axelsson and Thesleff 1958) by initiating calcium release from the intracellular stores (Weber and Herz 1968; Lüttgau and Oetlikar 1968; Geffner et al. 1975). The peak contracture tension is related to log caffeine concentration by an S-shaped curve. DES caused a shift of this curve towards lower caffeine concentrations (paper III). This finding can, to some extent at least, be explained on the basis of the above assumption that DES inhibits the SR calcium uptake during continued release by caffeine. As a result calcium is accumulated at the contractile sites and a contracture is produced by caffeine in a lower than normal concentration. This again is consistent with the idea that in skeletal muscle the mechanisms of calcium release and reuptake operate simultaneously.

It is possible that DES also inhibits the trans-membrane calcium influx, but in skeletal muscle such an action would not be expected to produce any noticeable effect, since the contractile activity in skeletal muscle is not immediately dependent on the extracellular calcium pool (Edman and Grieve 1964; Armstrong et al. 1972; Andersson and Edman 1974; for review see Fuchs 1974). Such an effect, would, on the other hand, be of relevance in muscles where the contractile response is directly dependent on trans-membrane influx of calcium (Edman and Schild 1962). This has indeed been verified in isolated uterine muscle where the contractile activity can be completely abolished by DES. The loss of contractility was found to be paralleled by a reduced calcium influx through the plasma membrane (Batra and Bengtsson 1978; Bengtsson 1978).

The contractile response of cardiac muscle is also very much dependent on the extracellular calcium pool (for review see Fozzard 1977). In accordance DES was found to reduce the twitch response of isolated papillary muscles, an effect which appears to be due to inhibition of trans-sarcolemmal influx of calcium by the drug (paper IV, and Khan and Wohlfart 1980).

The negative inotropic effect was not, however, accompanied by any significant prolongation of the relaxation phase (paper IV) as might be expected from the results obtained in skeletal muscle (see above). The explanation for this difference in results between cardiac and skeletal muscle might be found in the fact that, in cardiac muscle, SR amounts to about 0.3% of the total cell volume which is to be compared with a relative SR volume of 4.1% in skeletal muscle (Fabiato and Fabiato 1977). This suggests that in cardiac muscle, SR calcium uptake plays a minor role for relaxation. There may be other mechanisms of importance by which calcium is removed from the contractile machinery in the myocardium. Such mechanisms may include accumulation of calcium by cardiac sarcolemma as suggested by Lüllmann and Peters (1977; 1979) and Seibel et al. (1978). It seems that whatever cellular structure is responsible for cardiac muscle relaxation, DES has very little effect on its function.

A drug may affect the contractile response of a muscle fibre both by altering the kinetics of the action potential and by interfering with the intracellular calcium metabolism. The twitch enhancement by pentobarbital is accompanied by an increase in the initial rate of rise of tension and by a marked slowing of the relaxation phase. These mechanical changes are associated with an increase in the duration of the action potential. The prolongation of the action potential by pentobarbital would be expected to potentiate the twitch amplitude without influencing the initial rate of rise of tension (as is the case with 4-AP). An increase in the initial rate of force production, therefore, suggests that pentobarbital may enhance the rate of calcium release by a more specific action. This assumption is supported by the observation that the twitch depressing effect of dantrolene is reversed by pentobarbital, with a marked increase in the rate of force development (paper V). There is evidence that dantrolene suppresses the twitch response of the muscle fibre by inhibiting the release of activator

calcium from the intracellular stores (Van Winkle 1976; Desmedt and Hainaut 1977; Morgan and Bryant 1977). From this it is inferred that pentobarbital may enhance the rate of release of calcium in response to excitation. Increased rate of release of activator calcium occurring over a longer time (due to prolongation of the action potential) would result in a twitch potentiation accompanied by an increased rate of force development and a later attainment of peak tension. The inhibition of SR calcium uptake by pentobarbital would prolong the relaxation phase of the twitch as well as of tetanus (paper V) and would also contribute to the twitch potentiation (as in case of DES).

As discussed above DES and pentobarbital both seem to reduce the activity of the SR calcium pump which determines the time course of relaxation in skeletal muscle. However, the mechanism by which they inhibit this function is not understood. It is of interest to point out that Balzer et al. (1968) have suggested that drugs like reserpine, chlorpromazine and prenylamine inhibit SR calcium uptake by combining with unsaturated fatty acids of the SR lipid phase. This suggestion is supported by the finding that lecithin, which forms about 73% of the total SR lipids (Meissner and Fleischer 1971; Balzer and Khan 1975; Tada et al. 1978), contains a high percentage of 18-carbon chain unsaturated fatty acids (Balzer and Khan 1975). Furthermore it has already been shown that the presence of unsaturated fatty acids in the lipid phase of the SR membrane is important for the activity of calcium dependent ATPase (Fiehn and Hasselbach 1970; Warren et al. 1974; for review see Tada et al. 1978). It is, therefore, not unlikely that DES and pentobarbital, both being very lipid soluble, may readily penetrate the cell membrane and gain access to the SR membranes where they may exert their inhibitory action on the calcium pump by combining with unsaturated fatty acids of the SR lipids (c.f. above).

Ethanol in very high concentrations suppresses the twitch and tetanus amplitude of the single muscle fibre, with a marked decrease in the rate of tension development (paper VI). It is notable, however, that this effect of ethanol is associated with an increased duration of the action potential (paper VI). In such a situation one would expect potentiation instead of depression of the twitch amplitude. This discrepancy cannot be due to a block in the propagation of the action potential, since there was no apparent yielding at any place along the muscle fibre during contraction. However, it is possible that ethanol greatly reduces the release of calcium in response to the action potential. This would result in a decreased twitch amplitude accompanied by reduced rate of force development. The possibility was considered (paper VI) that the reduced twitch and tetanus response might be due to hypertonicity in presence of high ethanol concentration in the bathing solution. Previous studies have shown that in hypertonic solutions the contractile response of the muscle fibre is very much suppressed (Edman and Andersson 1968; Edman and Hwang 1977; for review see Ebashi 1976). Such an osmotic action may even be obtained with a relatively small molecule like urea (Edman, unpublished results). Ethanol, however, seems to produce no osmotic effect, since there was no apparent change in muscle fibre diameter. This is in line with the suggestion that ethanol readily equilibrates across the cell membrane and exerts no osmotic action (Abel 1980).

In contrast to ethanol, acetaldehyde potentiates the twitch response of the muscle fibre. The drug has no detectable effect on the initial rate of rise of tension nor does it affect the relaxation phase significantly (paper VI). The resting and action membrane potentials remain unaltered in the presence of acetaldehyde (paper VI). Therefore a mechanism similar to 4-AP does not seem to be involved in this case. The twitch depressing action of dantrolene is effectively counteracted by acetaldehyde suggesting

that the drug may potentiate the twitch by enhancing the release of calcium from the intracellular stores. In line with this view acetaldehyde in very high concentrations can induce contractures in the muscle fibre. In such high concentrations of acetaldehyde, however, the fibre is rendered inexcitable to electrical stimulus.

This study has provided evidence that drugs may affect similar basic mechanisms in different types of excitable cells but that the outcome of these effects depends on the physiological function of the mechanisms affected. 4-AP, for example, blocks the repolarizing potassium current in the membrane of nerve (Pelhate and Pichon 1974; Yeh et al. 1976), skeletal muscle (paper I, and Gillespie and Hutter 1975; Molg6 et al. 1977) and cardiac muscle (paper II, and Wollmer et al. 1979; Yanagisawa and Taira 1979) leading in all situations to an increase in the duration of the action potential (paper I and II, and Gillespie and Hutter 1975; Molg6 et al. 1977; Yeh et al. 1976; Wollmer et al. 1979; Yanagisawa and Taira 1979). In the case of the nerve terminal, transmitter is released when calcium enters the cell from the extracellular space during excitation (Katz and Miledi 1968). An increase in action potential duration therefore increases the intracellular calcium concentration resulting in an increased release of transmitter. In cardiac and skeletal muscle, prolongation of the action potential would increase the time during which activator calcium is released from the intracellular stores and this would lead to a potentiation of the twitch response. Furthermore, in cardiac muscle 4-AP by increasing the duration of the action potential would enhance the transmembrane calcium influx. This latter effect would increase the contractile state of the myocardial cell and may be the predominant factor for the positive inotropic action by 4-AP.

DES seems to inhibit the calcium movement across the sarcolemma and the calcium uptake by the sarcoplasmic reticulum in different types of

muscles. Inhibition of calcium influx at the level of plasma membrane would reduce the contractility of muscles that are critically dependent on the extracellular calcium pool as confirmed in uterine smooth muscle (Batra and Bengtsson 1978) and mammalian myocardium (paper IV, and Khan and Wohlfart 1980). In skeletal muscle, on the other hand, inhibition of calcium influx through the plasma membrane would be of minor significance as, in this case, extracellular calcium would seem to play no direct role in contractile activation. In skeletal muscle, calcium metabolism is mainly regulated by the sarcoplasmic reticulum (see above). Therefore an inhibition of the SR calcium pump by DES, in skeletal muscle, will lead to potentiation of the twitch and prolongation of the relaxation phase.

SUMMARY AND CONCLUSIONS

Effects of 4-aminopyridine (4-AP), diethyl-stilboestrol (DES), pento-barbital, ethanol and acetaldehyde were studied on the excitation-contraction coupling in frog isolated muscle fibres, rat whole lumbrical muscles and rabbit papillary muscles. Recordings included measurement of isometric twitch and tetanus responses, membrane action potentials and contractures produced by caffeine and increased potassium concentration.

4-AP increased the amplitude of the isometric twitch of frog single muscle fibres and rat lumbrical muscles. The rate of rise of tension was not changed. The rate of relaxation was reduced in the frog muscle fibres but was unaffected in the rat muscle preparation. The action potentials (only recorded in amphibian muscle fibres) showed a marked decrease in the rate of repolarization leading to a prolongation of the action potential. Experiments carried out in calcium free medium suggested that twitch potentiation by 4-AP was not due to increased trans-membrane calcium influx. The depressing effect of dantrolene on the contractility of the muscle fibre was counteracted by 4-AP. The contractile response produced by caffeine and by high potassium concentration was unaffected. The results are consistent with the view that twitch potentiation by 4-AP is due to increased release of calcium from the cellular stores caused by prolongation of the action potential. In frog muscle, 4-AP probably also reduces the rate of sequestration of activator calcium during the relaxation phase.

4-AP also potentiated the contractile response of the isolated papillary muscle. The total duration of the action potential was prolonged which was mainly attributable to slowing of the repolarizing phase. A mechanism involving greater calcium influx from the extracellular space into the myocardial calcium stores is likely to play a predominant role in the positive inotropic action of 4-AP.

DES potentiated the twitch response of the muscle fibre. The rate of rise of tension was not markedly affected but the relaxation phase of both twitch and tetanus was greatly prolonged. The drug caused no change of the action potential. From the results it is inferred that DES exerts an inhibitory action on the SR calcium pump. This would raise the concentration of the activator calcium at the contractile sites and cause increased twitch amplitude and decreased rate of relaxation. The idea that DES inhibits calcium resequestration is further supported by the finding that DES reduced the threshold concentration of caffeine required to produce contracture.

In contrast to the situation in skeletal muscle, DES greatly suppressed the contractile response of the isolated papillary muscle. The half time for relaxation and the duration of the action potential was not significantly affected. The results are consistent with the idea that DES, similar to its action in smooth muscle, reduces contractility in myocardium by inhibiting the trans-membrane calcium influx into the cell during excitation.

Pentobarbital in isolated skeletal muscle fibres increased the rate of force development as well as the total amplitude of the isometric twitch. It caused a moderate increase in the time to peak tension and markedly slowed the relaxation phase. The mechanical changes were associated with a prolongation of the action potential. The results suggest that twitch potentiation by pentobarbital is due both to increased rate of release of activator calcium and to extended time of release. An inhibitory action of the drug on SR calcium resequestration is likely to be responsible for the prolongation of the relaxation phase during twitch and tetanus.

The twitch response of the single muscle fibre was potentiated by acetaldehyde. The drug had no effect on the kinetics of the action potential that could account for the twitch potentiation. Acetaldehyde reversed the twitch depressant effect of dantrolene. In very high concentration,

acetaldehyde induced contractures. The results suggest that acetaldehyde potentiates the twitch response by enhancing the release of activator calcium from the cellular stores.

Ethanol in high concentrations greatly reduced both the rate of rise and total amplitude of force development during twitch and tetanus of single muscle fibres. The effects were associated with an increased duration of the action potential. Ethanol caused no detectable osmotic change that could account for the decreased mechanical output. It is suggested that the drug exerts an inhibitory action on the release of activator calcium during excitation.

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APPENDIX

- Paper I: Effects of 4-aminopyridine on the excitation-contraction coupling in frog and rat skeletal muscle.
- Paper II: Effects of 4-aminopyridine on contractile response and action potential of rabbit papillary muscle.
- Paper III: Effects of diethyl-stilboestrol on single muscle fibres of frog skeletal muscle.
- Paper IV: Effects of diethyl-stilboestrol on contractile response and action potential of rabbit papillary muscle.
- Paper V: Mechanism of action of pentobarbital on the contractile system of isolated frog muscle fibres.
- Paper VI: Influence of ethanol and acetaldehyde on electro-mechanical coupling of skeletal muscle fibres.

Effects of 4-aminopyridine on the excitation-contraction coupling in frog and rat skeletal muscle

By

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Abstract

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The effects of 4-aminopyridine (4-AP) were studied on isolated single muscle fibres of the frog and toe muscles of the rat. In both muscle preparations, 4-AP potentiated the twitch amplitude without significantly affecting the tetanus response. There was an increase of the time to peak tension and, in frog muscle, an increased time to half relaxation. 4-AP produced no change of the resting membrane potential. The rate of decay and, hence, the total duration of the action potential were markedly prolonged. 4-AP did not induce contractures by itself nor did it affect the contracture induced by caffeine. The mechanical threshold was determined by measuring the contracture response to various degrees of depolarization by potassium. This threshold was not affected by 4-AP. Twitch potentiation by 4-AP was independent of the extracellular calcium concentration. It is concluded that 4-AP potentiates the twitch response by increasing the release of activator calcium into the myofibrillar space by prolongation of the action potential. In addition, there may be a more direct inhibitory action of 4-AP on the calcium re-uptake by the sarcoplasmic reticulum in frog muscle.

Key words: 4-aminopyridine, skeletal muscle, single muscle fibre, excitation-contraction coupling, contractile potentiation, action potential, membrane potential

Recently interest has been focussed on the muscular effects of 4-aminopyridine (4-AP), an agent which has been shown to counteract the paralysis caused by curare (Paskov *et al.* 1973). The action of 4-AP on neuromuscular junction has been studied and there is evidence that this agent greatly potentiates transmitter release possibly by increasing the level of Ca^{2+} inside the nerve terminal (Molgo *et al.* 1975, 1977, Lundh and Thesleff 1977). However, little is known as to whether or not 4-AP also affects the contractile performance by acting directly on the excitation-contraction coupling. This latter aspect has been elucidated in more detail in the present study. To this end the effects of 4-AP on the time course of twitch and tetanus have been analyzed on single muscle fibres of the frog and on curarized toe muscles of the rat. Evidence will be presented to show that the twitch potentiation produced by 4-AP may at least partly be accounted for by prolongation of the membrane action potential.

Methods

Preparation

Frog muscle. Single muscle fibres were isolated from the ventral head of the semitendinosus muscle of *Rana temporaria*. For mounting the fibres, a link of stainless steel wire (thickness: 0.1 mm) was attached to each tendon as described previously (Edman and Kiessling 1971).

Rat toe muscle. Thin toe muscles were dissected from the front legs of Sprague-Dawley rats (150–200 g). A small loop of silk thread was tied to the tendon at each end of the muscle.

Muscle chamber

The preparations (frog single fibres or rat whole muscles) were mounted horizontally in a Perplex chamber between a tension transducer (see below) and an adjustable stainless steel hook, the position of which could be set by means of a micrometer screw. The chamber was 6 mm wide, 5 mm deep and contained 1.2 ml solution. The solution was changed by introducing fresh solution at the transducer end of the chamber, and it was removed by suction drain at the other end. A flush time of about 3 s was used when the potassium solutions were added for production of contractures. This caused an almost complete (>95%) exchange of the bathing solution (Andersson and Edman 1974 b).

Temperature control

During an experiment the temperature was controlled by a Colora Ultra-thermostat which circulated an ethylene glycol-water mixture through jackets surrounding the chamber and the containers of solution. The solution containers were connected with the chamber through polyethylene tubes (approximately 10 cm long) and a stopcock at the chamber inlet.

In studies on single muscle fibres, the bath temperature varied between 1 and 3.5°C and in studies on rat toe muscles between 22 and 23°C from expt. to expt. The temperature was maintained constant to within $\pm 0.2^\circ\text{C}$ throughout any particular expt., even during exchange of solution.

Tension recording

Tension was recorded by means of an RCA 5734 mechano-electric transducer. The signals were displayed on a Tektronix 502 A oscilloscope and were recorded simultaneously on paper by means of an Elema-Schönander ink-jet recorder.

Electrical stimulation

The single muscle fibre or the rat toe muscle was stimulated by means of a multielectrode assembly as described previously (Edman and Kiessling 1971). Pulses of 0.2 ms duration were used and the stimulation strength was adjusted to be supramaximal for each electrode pair. For tetanic stimulation, a 1 s train of pulses with a frequency of 16–20 Hz was used for the single muscle fibre. To obtain a fused tetanus in rat toe muscle, a 1 s train of pulses with a frequency of 100 Hz was used.

The single muscle fibre or the rat toe muscle was mounted for at least 1 h before the expt. and was tetanized at 10 min intervals during this time. During the actual expt. the preparation was stimulated every 2 min (if not otherwise stated) to record either twitch or tetanus response.

Solutions

Frog muscle fibre experiments. The solutions used had the following composition (mM):

Ordinary Ringer solution: NaCl 115.5, KCl 2.0, CaCl_2 1.8, Na-phosphate buffer 2.0, pH 7.0.

Calcium-free Ringer: NaCl 116.3, KCl 2.0, EDTA 1.0, Na-phosphate buffer 2.0, pH 7.0.

Lanthanum containing Ringer: NaCl 117.2, KCl 2.0, La 0.05, Tris-(hydroxymethyl)-aminomethane 2.0. The pH was adjusted to 7.0 by addition of H_2SO_4 to a final concentration of 0.94 mM.

For potassium contractures, solutions of the following composition were used:

Solution (mM)	KCl	K- CH_3SO_4	Na- CH_3SO_4	NaCl	CaCl_2
10 K-Ringer	10	—	96.90	10.60	1.80
20 K-Ringer	—	20	88.99	8.51	1.80
40 K-Ringer	—	40	75.05	2.45	1.80
80 K-Ringer	—	80	37.80	—	1.50
117.5 K-Ringer	—	117.5	0.77	—	1.03

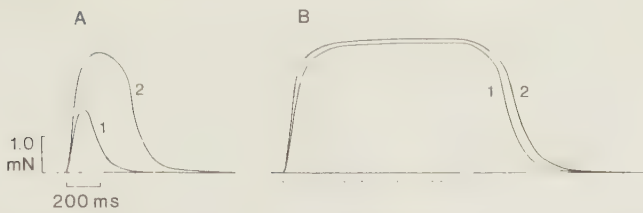


Fig. 1. Effects of 4-AP on isometric twitch (A) and tetanus (B) in frog single muscle fibre. 1. Control, normal Ringer solution. 2. In the presence of 3 mM 4-AP. Stimulus markers below base line.

All K-Ringer solutions contained 2 mM Tris-(hydroxymethyl)-aminomethane. The pH was adjusted to 7.0 by addition of H_2SO_4 to a final concentration of 0.94 mM.

Rat toe muscle experiments. Tyrode solution (mM): NaCl 154.0, KCl 5.6, NaHCO_3 3.6, CaCl_2 1.0, NaH_2PO_4 0.55, Na_2HPO_4 0.9, glucose 5.5, 0.5 mg/ml of d-tubocurarine. This solution was aerated by a mixture of 95% O_2 and 5% CO_2 .

Drugs. 4-aminopyridine (4-AP), caffeine and dantrolene were dissolved in Ringer and Tyrode solutions, respectively.

Caffeine contractures in frog muscle fibres

Caffeine solutions of the following concentrations were used (mM): 1.0, 1.5, 2.0, 3.0 and 5.0. The solutions were flushed from a pre-cooled container until plateau contracture tension was achieved. This occurred after approximately 15 s. Relaxation was produced by re-introduction of the normal Ringer solution. The time interval between two successive potassium or caffeine contractures was 20–25 min.

Recording of membrane potential

Conventional glass capillary electrodes (about 20 M Ω) filled with 2.5 M KCl were used for intracellular recordings of membrane potentials. The reference electrode was an Ag-AgCl electrode connected to the bath through an agar-Ringer bridge. The microelectrode and the reference electrode were connected via an electrometer provided with capacitance neutralization to a Tektronix 502A oscilloscope. The signals were photographed on 35 mm film. The amplified signals were also used to modulate an audio frequency generator. Successful impalement was indicated by an abrupt change in frequency. When action potentials were recorded the fibre was stimulated only at one locus, approximately 5 mm from the tip of the micro-electrode.

Results

A. Frog muscle fibres

Twitch and tetanus responses. 4-aminopyridine (4-AP) was tested on isolated muscle fibres in concentrations ranging between 0.02 and 3.0 mM. In a concentration of 1 mM or higher, 4-AP potentiated the twitch response without significantly affecting the tetanus response. Maximum twitch potentiation was obtained by 3 mM 4-AP (Fig. 1). The magnitude of the twitch potentiation was inversely related to the twitch/tetanus ratio recorded in the control Ringer solution. At full effect of 4-AP a peak twitch force of more than 90% of the tetanic tension was generally attained.

As illustrated in Fig. 1, 4-AP caused no significant change in the initial rate of rise of tension. The increase in twitch amplitude was associated with an increase of the time to peak tension and a prolongation of the relaxation phase. With 3 mM 4-AP the half time to peak tension and the half time for relaxation increased by 95% and 74% of the control values, respectively (Table I).

TABLE I. Effects of 4-AP on twitch parameters in frog single muscle fibres. Each value represents the mean of 5–10 twitch responses.

Expt. no.	Normal Ringer		3 mM 4-AP in Ringer		T_A/T_C	R_A/R_C
	Half time to peak tension (ms) T_C	Half time for relaxation (ms) R_C	Half time to peak tension (ms) T_A	Half time for relaxation (ms) R_A		
1	85	130	142	210	1.67	1.62
2	86	144	180	263	2.09	1.83
3	85	165	177	285	2.08	1.73
4	76	172	148	230	1.95	1.34
5	69	84	136	184	1.97	2.19
Grand mean	80	139	157	234	1.95	1.74

The effect produced by 4-AP was only partially reversible. As illustrated in Fig. 2, repeated washing of the fibre in normal Ringer lowered the amplitude of the potentiated twitch slightly. When 3 mM 4-AP was re-introduced the twitch amplitude was again increased.

To test if the extracellular calcium plays any role in the twitch potentiation produced by 4-AP, calcium was removed from the extracellular medium by repeated washing of the fibre with a calcium-free Ringer solution containing 1 mM EDTA. Fig. 3 b shows, in confirmation of previous results (Edman and Grieve 1961, 1964, Jenden and Reger 1963, Curtis 1963), that removal of calcium from the extracellular medium made the fibre inexcitable. Addition of lanthanum to the bathing solution in a concentration (0.05 mM) sufficient to normalize the resting membrane potential in the absence of calcium (Andersson and Edman 1974 a) restored the twitch response (Fig. 3 c). 4-AP (3 mM) added to the calcium-free lanthanum-containing medium caused a potentiation of the twitch in much the same way as in normal Ringer (Fig. 3 d).

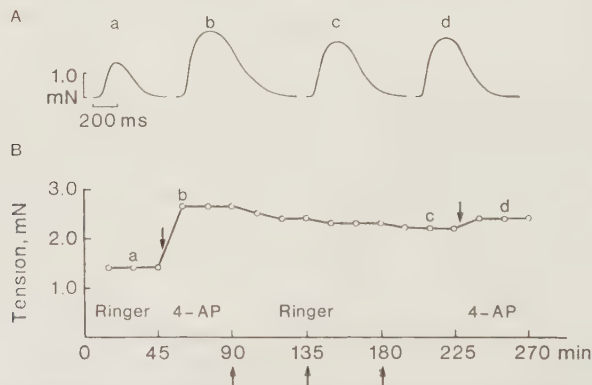


Fig. 2. Reversibility of the effects produced by 4-AP on the twitch response of frog muscle fibre. Tracings in A refer to times indicated in B. Arrows above curve in B indicate addition of 3 mM 4-AP. Arrows below abscissa indicate washings with Ringer solution. Note that twitch potentiation is only partially reversible.

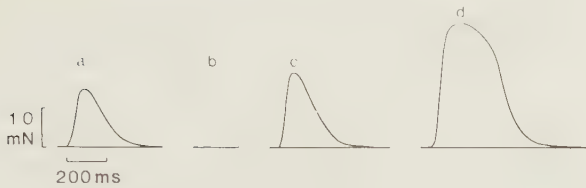


Fig. 3. Effects of 4-AP on twitch response of frog muscle fibre after removal of calcium from the extracellular medium. (a) Normal Ringer. (b) Approximately 5 min after removal of calcium (1 mM EDTA present). No twitch response. (c) After replacing calcium with 0.05 mM lanthanum. (d) Effects of 3 mM 4-AP in calcium free Ringer containing 0.05 mM lanthanum.

Dantrolene has a powerful depressant effect on the isometric twitch. In relatively low concentrations, it greatly suppresses the twitch response of single muscle fibres (Ellis and Brayant 1972, Hainaut and Desmedt 1974). The effect of dantrolene on 4-AP-treated fibres was studied. As illustrated in Fig. 4, dantrolene reduced the 4-AP potentiated twitch.

Potassium and caffeine induced contractures. Experiments were performed to find out if 4-AP affected the mechanical threshold, *i.e.* the membrane potential at which contractile activation is initiated. For this purpose contractures were produced by immersing the single fibre in an isotonic solution containing potassium (constant $[K]$, $[Cl]$ product) in concentrations varying between 10 and 117.5 mM. Peak contracture tension was plotted against $\log [K]$. Fig. 5 summarizes the results obtained in three experiments, in which the effect of 3 mM 4-AP was tested. There was a very steep rise of the contracture response in the range 10–20 mM potassium. As can be seen, 4-AP did not cause any significant change of the fibre's response to potassium depolarization.

Similarly, the effect of 4-AP on caffeine induced contractures was studied. The peak tension developed was plotted against $\log$ caffeine concentration. The S-shaped curve relating contracture tension and caffeine concentration also remained unaltered in the presence of 3 mM 4-AP (Fig. 6).

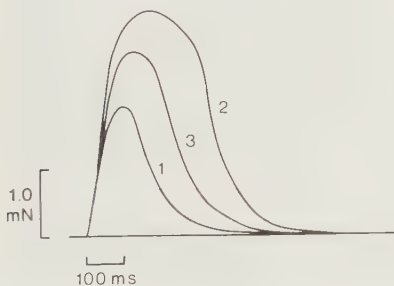


Fig. 4

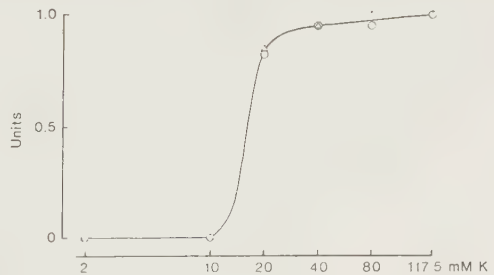


Fig. 5

Fig. 4. Effects of dantrolene on the twitch response of frog muscle fibre in the presence of 3 mM 4-AP. 1. Normal Ringer. 2. In the presence of 4-AP. 3. In the presence of 4-AP and 9 μ M dantrolene.

Fig. 5. Relation between peak contracture tension and extracellular potassium concentration in the presence and absence of 4-AP. Frog muscle fibres. $\circ$: Control, no 4-AP. Δ : In the presence of 3 mM 4-AP. Each point represents mean value of three different experiments.

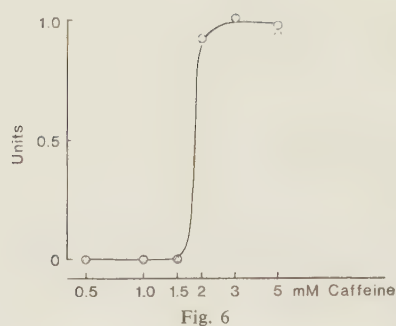


Fig. 6

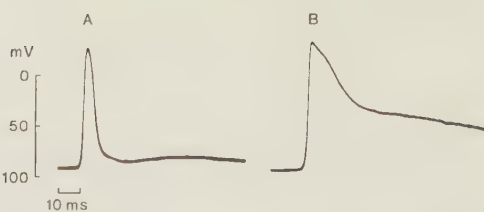


Fig. 7

Fig. 6. Relation between peak contracture tension and caffeine concentration in the presence and absence of 4-AP. Frog muscle fibres. $\circ$: Control, no 4-AP. $\triangle$: In the presence of 3 mM 4-AP. Each point represents mean value of three different experiments.

Fig. 7. Effects of 4-AP on intracellularly recorded action potential of frog single muscle fibres. A. Normal Ringer. B. In the presence of 3 mM 4-AP. The records in A and B are from different fibres.

Resting and action potentials. 4-AP in a bath concentration of 3 mM did not affect the resting membrane potential over at least 2 hours as tested in 13 fibres of altogether 6 fibre bundles (Table II). The effects of 4-AP on membrane action potential were studied after full twitch potentiation had developed. There were no significant changes of the maximum rate of rise and overshoot of the action potential. The maximum rate of fall and the duration of the action potential, on the other hand, were both markedly prolonged (Fig. 7 and Table II).

B. Rat toe muscle

Experiments were performed to study the effects of 4-AP on twitch and tetanus responses of mammalian skeletal muscle. Curarized toe muscles of the rat were stimulated at 22–23°C to produce a single twitch or a 1 s fused tetanus at 2 min intervals. 4-AP in a concentration ≥ 0.1 mM caused potentiation of the isometric twitch (Fig. 8). The peak twitch amplitude was increased by approximately 1/3 of the control in response to 0.1 mM 4-AP. Similar to the situation in frog skeletal muscle 4-AP prolonged the time to peak twitch tension. However,

TABLE II. Effects on resting and action potentials of 3 mM 4-AP added to normal Ringer (Mean $\pm$ S.E.). Frog muscle fibre bundles exposed to 4-AP for 15–20 min before recording the resting and action potentials. Number of fibres for each measurement given within brackets. Students' *t*-test: + + +, $P < 0.001$.

	Resting potential mV	Action potential			
		Overshoot, mV	Maximum rate of rise, V/s	Maximum rate of decay, V/s	Duration at -25 mV level, ms
Control	84.1 $\pm$ 2.3 (13)	33.7 $\pm$ 2.2 (10)	56.0 $\pm$ 3.5 (10)	26.4 $\pm$ 1.8 (10) + + +	5.2 $\pm$ 0.2 (10) + + +
4-AP (3 mM)	87.6 $\pm$ 1.8 (13)	25.5 $\pm$ 3.3 (7)	55.9 $\pm$ 4.6 (7)	4.3 $\pm$ 0.4 (7)	16.2 $\pm$ 1.6 (7)

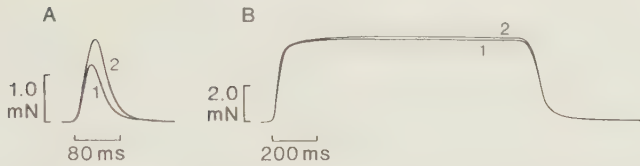


Fig. 8. Effects of 4-AP on isometric twitch (A) and tetanus (B) of a curarized toe muscle of the rat. 1. Normal Tyrode solution. 2. In the presence of 0.1 mM 4-AP. Note different time and tension scales.

the time to 50% relaxation in the rat toe muscle was not significantly affected (Table III). There was no clear effect of 4-AP on the amplitude of the fused tetanus when care was taken to stimulate the muscle supramaximally. When submaximal stimulation strengths were used, 4-AP in concentrations >0.1 mM reduced the tetanic force. This suggests that 4-AP raised the electrical threshold thereby reducing the number of fibres activated under these conditions.

Discussion

Previous experiments (Lundh *et al.* 1977) performed on the rat extensor digitorum longus muscle suggest that 4-amino-pyridine (4-AP) potentiates muscle contraction by facilitating transmitter release at the neuromuscular junction and the drug has been shown to be a powerful antagonist of the neuromuscular blocking agent botulinum toxin (Lundh *et al.* 1977, Lundh and Thesleff 1977). The present results on frog single muscle fibres and on curarized rat toe muscles demonstrate that 4-AP causes twitch potentiation by a more direct action on the excitation-contraction coupling. In order to produce this effect, however, considerably higher concentrations were required than those needed to affect the transmitter release (Lundh 1978).

The twitch potentiation by 4-AP is characterized by an increased peak amplitude and an increase of the half time to peak tension and of the half time for relaxation. These changes were associated with a broadening of the action potential. As previously demonstrated on single muscle fibres (Edman *et al.* 1966) and in experiments on whole muscle (Sandow and Preiser 1964, Sandow, Taylor and Preiser 1965, Taylor *et al.* 1972) the duration of the mechanical activity appears to be quantitatively related to the action potential duration (at the -25 mV level). This has been suggested to mean, that the action potential governs

TABLE III. Effects of 4-AP (0.1 mM) on the twitch parameter of curarized toe muscles of rat. Each value represents the mean of 3-4 responses.

Expt. no.	Normal Ringer		3 mM 4-AP in Ringer		T_A/T_C	R_A/R_C
	Half time to peak tension (ms) T_C	Half time for relaxation (ms) R_C	Half time to peak tension (ms) T_A	Half time for relaxation (ms) R_A		
1	14	30	19	29	1.36	1.03
2	12	17	14	17	1.17	1.00
3	15	23	16	20	1.07	0.87
Grand mean	14	23	16	22	1.20	0.97

the time during which activator Ca^{2+} is released into the myofibrillar space (Sandow *et al.* 1964, Edman *et al.* 1966, Taylor *et al.* 1972). The longer duration of the action potential would cause a higher peak concentration of Ca^{2+} at the contractile sites, and this in turn would require a longer time for the elimination of Ca^{2+} from the myofibrils. If the peak concentration of the activator Ca^{2+} were large enough to fully activate the contractile system, a further increase of the Ca^{2+} concentration would merely cause a prolongation of the mechanical activity. This would be reflected in the isometric twitch response as an increased peak amplitude and a later attainment of peak tension, whereas the initial rate of the rise of the twitch tension would remain unaffected. The effects produced by 4-AP are fully consistent with the idea that twitch potentiation is due to the prolongation of the action potential according to the above mechanism. However, the results clearly show that 4-AP also prolongs the relaxation time in frog muscle suggesting that 4-AP exerts a more direct inhibitory action on the resequestration of calcium in this tissue.

It is of interest to note in this connection that 4-AP does not affect the mechanical threshold, *i.e.* the potential level at which contraction is initiated in response to membrane depolarization. This is inferred from the finding that 4-AP does not shift the curve relating potassium concentration and contracture response (Fig. 5).

Caffeine and 4-AP affect the time course of the isometric twitch in a similar way. Previously it was considered that the effect of caffeine is due to an inhibitory action on the calcium pump of the sarcoplasmic reticulum (SR) (Weber 1968). This view has recently been revised by Endo (1975) who suggests that caffeine potentiates the twitch response, and induces contractures in higher concentrations, by enhancing the calcium-induced calcium release from the SR. Such a mechanism of action does not seem to operate in twitch potentiation by 4-AP, as this agent, even in high concentrations (10 mM), fails to induce caffeine-like contractures and has no detectable influence on the contractures induced by caffeine (Fig. 6).

From the results presented in Table II it is evident that 4-AP does not cause any detectable change of the resting membrane potential. The maximum rate of rise and the overshoot of the action potential were not significantly different from the control. The rate of decay of the action potential, on the other hand, was markedly reduced. These changes of the action potential are consistent with previous observations which suggest that 4-AP acts by predominantly decreasing the potassium conductance in frog skeletal muscle fibres (Gillespie and Hutter 1975) and in cockroach (Pelhate and Pichon 1974) and squid (Yeh *et al.* 1976) axon membranes and in sympathetic nerves (Kirpekar *et al.* 1977).

In contrast to its effects on the nerve terminal (Lundh *et al.* 1977) 4-AP had a slower onset of action on the muscle fibres, 15–20 min being required for the full effect to appear. Furthermore the effects produced by 4-AP on the action potential and on the twitch kinetics were only partially reversible. This is consistent with the idea that there is a very firm binding of 4-AP both to the surface membrane and, in frog muscle, to the sarcoplasmic reticulum inside the fibre (*cf.* above).

It has been suggested that 4-AP increases transmitter release from nerve endings by facilitating calcium influx from the extracellular medium (Molgo *et al.* 1977, Lundh and Thesleff 1977). The present results suggest, however, that extracellular calcium is immaterial for the twitch potentiation produced by 4-AP. The enhancement of the isometric twitch

by 4-AP could thus be produced when there was no calcium in the extracellular medium, *i.e.* after replacement of calcium in the Ringer solution by lanthanum. It has previously been shown that the presence of lanthanum (Andersson and Edman 1974 a) prevents the membrane depolarization that otherwise occurs as calcium is removed from the bathing fluid (Edman and Grieve 1961, 1964, Jenden and Reger 1963, Curtis 1963). Lanthanum is unlikely to penetrate the fibre membrane (Laszlo *et al.* 1952, Lesseps 1967, Langer and Frank 1972) and causes by itself only a slight twitch potentiation (Andersson and Edman 1974 a) in the low concentrations (0.05 mM) used here.

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Effects of 4-aminopyridine on contractile response and action potential of rabbit papillary muscle

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The effects of 4-aminopyridine (4-AP) were studied on isolated papillary muscles from the heart of reserpinized rabbits (at 37°C). The preparations were paced to contract at 0.67 Hz under isometric conditions and the muscle length was adjusted to 95% of the length for optimum force production. Simultaneous recordings of isometric force and membrane potentials were performed. 4-AP (50 µM) increased peak force by approximately 20% of the control and prolonged the action potential by 20%. Higher concentrations of 4-AP (800 µM) resulted in further increments of force and action potential duration (60 and 70% of controls, respectively). Prolongation of the action potential and potentiation of the isometric force was still present one hour after withdrawal of the drug from the perfusate. The results are consistent with the view that 4-AP prolongs the action potential by inhibiting the late repolarizing potassium current. It is suggested that the calcium uptake by the ventricular cell during the prolonged action potential is increased and that this leads to the positive inotropic effect.

Key words: 4-aminopyridine, myocardium, calcium metabolism, action potential, contractility, excitation-contraction coupling.

INTRODUCTION

The aminopyridines form a group of pharmacological compounds which interfere with the process of transmitter release at chemical synapses (Thesleff 1980). 4-aminopyridine (4-AP) in micromolar concentrations markedly potentiates the release of acetylcholine at the neuromuscular junction (Molgo, Lemeignan & Lechat 1975, 1977, Lundh & Thesleff 1977), and this compound has been used in man to improve neuromuscular transmission (Lundh, Nilsson & Rosén 1977, 1979, Yong, Goldner & Sanders 1980). The release of noradrenalin from synaptic nerve terminals in various organs is also facilitated by 4-AP (Johns et al. 1976, Kirpekar, Kirpekar & Prat 1977, Yanagisawa, Satoh & Taira 1978, Weide & Löffelholz 1980). These effects have been attributed to an increased influx of calcium into the nerve terminal during a prolonged action potential (Molgo, Lemeignan & Lechat 1975, 1977, Lundh & Thesleff 1977, Kirpekar, Kirpekar & Prat 1977, Illes & Thesleff 1978, Lundh 1978).

4-AP in higher concentrations (about 3 mM) has direct effects on the excitation-contraction process in skeletal muscle (Khan & Edman 1979), the twitch response being greatly potentiated both in amphibian and in mammalian muscle fibres. The action potential of the muscle fibres is also increased in duration. The potentiation of the twitch has been attributed to an enhanced calcium release from the sarcoplasmic reticulum during excitation.

Changes in the inotropic state of cardiac muscle is generally attributed to variations in transmembrane calcium fluxes and intracellular distribution of calcium (Fozzard 1977, Fabiato & Fabiato 1979). Since 4-AP exhibits prominent effects on the cellular handling of calcium, it was of interest to investigate the effect of this agent on cardiac muscle. Preliminary results of this study have been reported previously (Wollmer, Wohlfart & Khan 1979).

METHODS

Preparation and mounting. Papillary muscles (length 2-4 mm, diameter 0.4-0.8 mm) were dissected from the right ventricle of small (weight about 1 kg) rabbits. All animals were reserpinized (Serpasil^R, CIBA, 4 mg/kg) 4-5 hours before the killing. The preparation was mounted horizontally in a thermostatically controlled bath (36.5-37.5°C) between a force transducer and a steel hook (for details of the preparation see Wohlfart, Grimm & Edman 1977). The muscle was stimulated to contract isometrically at a rate of 0.67 Hz by passing a current from a Grass S-44 stimulator between two platinum electrodes placed on either side of the muscle. The stimulus pulse was a unidirectional square wave of 2 ms duration and with an amplitude of about 150% of the threshold value. The length of the muscle was adjusted to 95% of the length of optimum force production.

Solutions. The bath was continuously perfused (flow rate about 4 ml/min) with oxygenated (95% O₂ and 5% CO₂) solution of the following composition (mM): NaCl 100, KCl 4.0, MgSO₄ 1.5, CaCl₂ 2.0, NaHCO₃ 20.0, NaH₂PO₄ 1.5, Na-acetate 20.0 and glucose 10.0. The solution also contained insulin, 2 U/l. The pH of the solution was 7.4. All chemicals were of analytical grade and dissolved in double-distilled water. 4-aminopyridine sulfate was added to the solution in a range of concentrations.

Recording of mechanical and electrical responses. Isometric force was recorded with a strain-gauge force transducer. Conventional glass capillary electrodes filled with 2.5 M KCl and with a resistance of 10-30 Mohm were used to record the membrane potential. Action potentials and myograms were recorded by an ink-jet writer (Elema-Schönander myogograf) at a low paper speed. Selected recordings were displayed on a Tektronix 5103N storage oscilloscope and photographed on 35 mm orthochromatic film.

Film analysis. Measurements of the oscilloscope records were made at 10X magnification in a Nikon 6C profile projector. The Stage micrometer readings were used to calculate the amplitude of the myogram. The action potential duration was defined as the time from the stimulus to the repolarization of the action potential at a voltage of -40 mV.

RESULTS

The effect of 4-AP (200 μ M) on the isometric myogram of an isolated papillary muscle is shown in Fig. 1. The myograms shown in this figure were continuously recorded and superimposed during the first 20 minutes of exposure to 4-AP. It can be seen that 4-AP increased the rate of rise of force and the maximum amplitude of the contraction. Time to peak force was prolonged. There was, however, no effect on the resting force of the preparation.

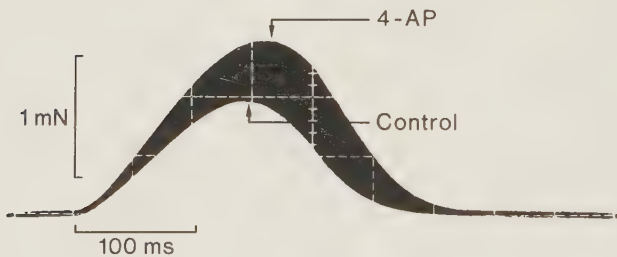


Fig. 1. The effect of 200 μ M 4-AP on the isometric twitch of an isolated papillary muscle. The myograms were recorded continuously and superimposed during the first 20 minutes of exposure to 4-AP.

Fig. 2 demonstrates the effect of 4-AP on the action potential. Simultaneously recorded myograms are also shown. A prolongation of the action potential associated with the positive inotropic effect is evident.

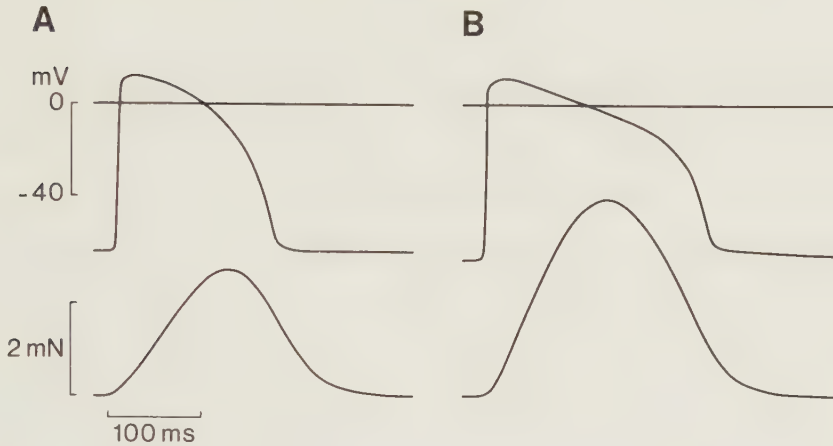


Fig. 2. Potentiation of the isometric twitch (lower panels) and prolongation of the action potential (upper panels) by 4-AP. A control solution. B 100 μ M 4-AP.

The lowest concentration of 4-AP that produced significant effects in our preparations was 25-50 μ M. Dose-response curves for the effects of 4-AP on contraction amplitude and action potential duration are given in Fig. 3. Peak force increased from about 120 to about 160% of the control value when the concentration of 4-AP was raised from 50 to 800 μ M. The action potential duration increased from about 120 to about 170% of the control value over the same range of concentrations.

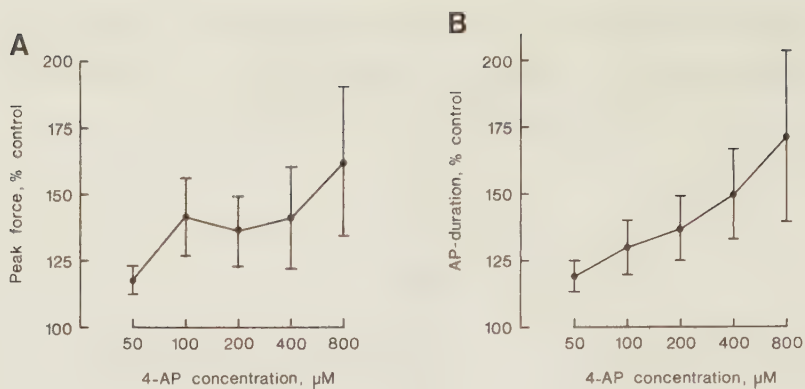


Fig. 3. Peak force (A) and action potential duration (B) in relation to the concentration of 4-AP in the perfusate. Each value represents the mean ($\pm$ S.E.) of five reserpinized preparations. Five measurements were obtained in at least three different cells in each preparation at the various concentrations of 4-AP.

Fig. 4 shows the effect of 4-AP in a high concentration - 4 000 μM . This is in the range of concentrations giving a potentiation of the single twitch in skeletal muscle fibres (Khan & Edman 1979). As is seen in the figure, the action potential of the papillary muscle was very much prolonged and was actually of longer duration than the twitch. Furthermore, relaxation occurred during the plateau phase of the action potential.

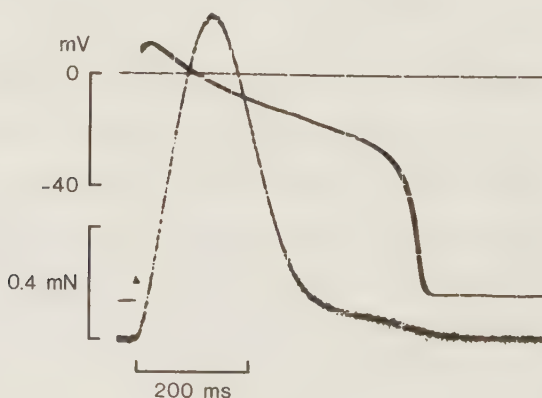


Fig. 4. Effect of 4 000 μM 4-AP on the isometric twitch and action potential. Note that relaxation occurs during the plateau phase of the action potential.

The effects of 4-AP on the ventricular preparation were not easily reversible. After exposure to 4-AP and subsequent change to control solution, peak force and action potential duration gradually decreased, but control values were generally not reached within one hour.

DISCUSSION

Preliminary studies (Wollmer, Wohlfart & Khan 1979) showed that 4-AP exerts a positive inotropic effect on isolated papillary muscle of the rabbit heart. Similar findings were also reported by Yanagisawa & Taira (1979) using trabecular muscle of dog heart. The positive inotropic effect probably results from 4-AP induced noradrenaline release from sympathetic nerves within the preparation (Johns et al. 1976, Kirpekar, Kirpekar & Prat 1977, Yanagisawa, Satoh & Taira 1978, Yanagisawa & Taira 1979, Weide & Löffelholz 1980) as well as from a direct effect on the excitation-contraction process in the myocardium. In the present study, the animals were reserpinized in an attempt to study the direct effect only. The inotropic effect was accompanied by an increased duration of the action potential. Both the inotropic effect and the lengthening of the action potential increased with the bath concentration of 4-AP in the range 50-800 μ M.

It seems likely that the positive inotropic effect is a consequence of increased calcium uptake by the ventricular cell. This increase in calcium uptake could be explained by an intensified calcium current during excitation. Another possibility is that calcium influx occurs over a longer period of time during each prolonged action potential. Either of these mechanisms would result in a greater content of calcium in the cellular stores (cf. Wohlfart 1979). More calcium would, therefore, be released upon excitation, yielding contractile potentiation. Increased calcium uptake during a prolonged action potential, induced by 4-AP, has previously been suggested to explain the increase in transmitter release from nerve endings (Molgo, Lemeignan & Lechat 1975, Kirpekar, Kirpekar & Prat 1977, Lundh & Thesleff 1977, Lundh 1978).

Another consequence of the prolongation of the action potential would be a longer time for release of activator calcium from the intracellular stores. In the isometric twitch, this would be reflected as a longer time to peak force (Figs. 1, 2). A similar mechanism has been suggested to explain the potentiating effect of 4-AP on the twitch amplitude in skeletal muscle fibre (Khan & Edman 1979) and in rat portal vein (Leander, Arner & Johansson 1977).

The increased duration of the myocardial action potential may be due to a decrease of the repolarizing potassium current. This is in keeping with previous studies on nerve and skeletal muscle preparations (Pelhate & Pichon 1974, Yeh et al 1976, Gillespie & Hutter 1975).

Voltage clamp experiments suggest (Morad & Goldman 1973) that the time for release of calcium from cellular stores in cardiac muscle can only be increased up to a certain limit by prolongation of the depolarizing pulse. There is also, however, a reuptake of calcium within the cell, leading to the relaxation. This combination of a time-limited release and a reuptake of calcium may explain why, in high concentration of 4-AP, relaxation occurs during the plateau phase of the very prolonged action potential (Fig. 4). This finding also gives some support to the idea that calcium is released from an intracellular store at excitation (Edman & Jóhannsson 1976, Wohlfart 1979) rather than being transported directly from the interstitium to the myofilaments during the plateau phase of the action potential.

The relatively long lasting effect of 4-AP (about one hour) could be explained by an intracellular site of action of the drug (cf. Horn, Lambert & Marshall 1979, Molgo, Lundh & Thesleff 1980). Experiments on isolated skeletal muscle fibres have shown that repeated stimulation of the muscle fibre exposed to 4-AP is required in order to obtain potentiation of the twitch (Khan & Edman, unpublished observations). This suggests that the drug must enter the sarcolemma in order to produce its effects.

In conclusion, we suggest that 4-AP acts primarily on the sarcolemma. It enhances calcium influx and prolongs the action potential, leading to greater calcium contents in cellular stores and a positive inotropic effect.

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Effects of diethyl-stilboestrol on single fibres of frog skeletal muscle

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The effects of diethyl-stilboestrol (DES), one of the most potent estrogens, were studied on single muscle fibres of the frog. In relatively low concentrations ($5 \mu\text{M}$), DES greatly potentiates the twitch response of the fibre without significantly affecting the amplitude of the tetanus response. The twitch potentiation is accompanied by an increase in time to peak tension and a marked prolongation of the relaxation phase. In tetanic response the half decay time after the last stimulus is also prolonged by DES. DES causes no changes in the resting or action potential of the muscle fibre. The S-shaped curve relating peak contracture tension and caffeine concentration is shifted towards lower caffeine concentrations by DES. Dantrolene greatly suppresses the DES potentiated twitch. It is concluded that DES potentiates the twitch response and prolongs the relaxation time by inhibition of the calcium re-uptake by the sarcoplasmic reticulum.

Key words: Diethyl-stilboestrol, skeletal muscle, single muscle fibre, excitation-contraction coupling, contractile potentiation, action potential, membrane potential

Estrogens are known to influence the contractile activity of uterine muscle (Bengtsson 1973, Fuchs 1974, Batra & Bengtsson 1978). Their precise mechanism of action, however, is not well understood. Previous studies suggest that diethyl-stilboestrol (DES), one of the most potent estrogens, influences the contractile activity of the uterus by inhibiting mitochondrial calcium uptake (Batra & Bengtsson 1972).

In skeletal muscle intracellular calcium concentration and thus the contractile activity of the muscle (for review see Sandow 1965) is regulated by a well organised sarcoplasmic reticulum (SR). It was therefore of interest to study the effects of DES on skeletal muscle. Experiments were performed on single muscle fibres. Evidence will be presented showing a great potentiation of the twitch response by DES. Increase in twitch amplitude is associated with a marked prolongation of the relaxation phase suggesting an inhibitory action of DES on SR calcium uptake.

METHODS

Experiments were performed on single muscle fibres from the ventral head of the semitendinosus muscle of *Rana temporaria*. Methods used for mounting, stimulation, temperature control and caffeine contractures were essentially similar to those described previously (Khan & Edman 1979).

Recording of membrane potential. The resting and action potentials were recorded, in the presence and absence of $5 \mu\text{M}$ DES, using conventional glass capillary electrodes (Khan & Edman 1979).

In these experiments the bath temperature varied between 2.5 and 5.0°C from expt. to expt., but was maintained constant to within $\pm 0.2^\circ\text{C}$ throughout any particular expt., even during exchange of solutions.

Solutions. A stock solution (10 mM) of diethyl-stilboestrol (DES) was prepared in absolute alcohol from which 2 , 5 and $10 \mu\text{M}$ solutions in Ringer were prepared. Maximum alcohol concentration was 1 ml/l of Ringer solution. A $9 \mu\text{M}$ solution of dantrolene sodium was prepared in Ringer solution.

All chemicals used were of analytical grade and dissolved in double distilled water. Dantrolene sodium was obtained from Eaton Chemicals, Norwich, N.Y., U.S.A. and diethyl-stilboestrol was from Sigma Chemical Co., St. Louis, U.S.A.

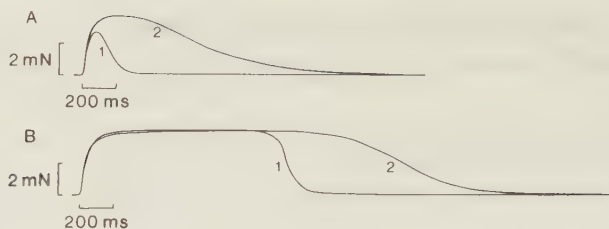


Fig. 1. Effects of DES on isometric twitch (A) and tetanus tension (B) in frog single muscle fibre. % Control, normal Ringer solution. 2. In the presence of $5 \mu\text{M}$ DES. Stimulus marker below baseline.

RESULTS

Twitch and tetanus response. Effects of diethylstilboestrol (DES) were studied on single muscle fibres in concentrations ranging between 2 and $10 \mu\text{M}$. In a concentration of $2 \mu\text{M}$ or higher, DES greatly potentiated the twitch response without significantly affecting the amplitude of the tetanus (Fig. 1 A and B). Maximum potentiation was obtained by $5 \mu\text{M}$ DES. Twitch potentiation was inversely related to the twitch/tetanus ratio recorded in control Ringer solution.

As illustrated in Fig. 1 A and B, DES caused no change in the initial rate of rise of tension neither in twitch nor in tetanus response. In the twitch response the increase in amplitude was associated with an increase in time to peak tension and a very pronounced prolongation of the relaxation phase (Fig. 1 A). In tetanus response DES also caused a prolongation of the relaxation time after the last stimulus (Fig. 1 B). The effects produced by DES were not reversible. Repeated washing of the fibre with Ringer only lowered the amplitude of the potentiated twitch slightly.

In concentrations $10 \mu\text{M}$ or higher of DES, the fibre became irreversibly inexcitable to the electrical stimulus. No contractions were induced by DES even when a $25 \mu\text{M}$ concentration was used.

The effect of DES on caffeine induced contractions was also studied. The peak tension developed, before and after DES ($5 \mu\text{M}$), was plotted against log caffeine concentration. As illustrated in Fig. 2, DES shifted the S-shaped curve relating the contracture tension and caffeine concentration to the left.

Dantrolene is known to have a powerful depressant effect on the isometric twitch. In relatively low concentrations ($10 \mu\text{M}$) it greatly suppresses the

twitch response of single muscle fibres (Hainaut & Desmedt 1974, Edman 1979). The effect of dantrolene on DES treated fibres was studied. As illustrated in Fig. 3, dantrolene ($9 \mu\text{M}$) almost normalized the DES potentiated twitch.

Membrane potential. DES in a bath concentration of $5 \mu\text{M}$, which caused maximum twitch potentiation, did not affect the resting membrane potential over at least 2 h as tested in 15 fibres of altogether 5 fibre bundles. From Fig. 4 and Table 1 it is evident that DES neither caused any significant change in the maximum rate of rise and decay nor in the overshoot of the action potential.

DISCUSSION

In a muscle fibre inhibition of the calcium uptake by the sarcoplasmic reticulum (SR) would increase

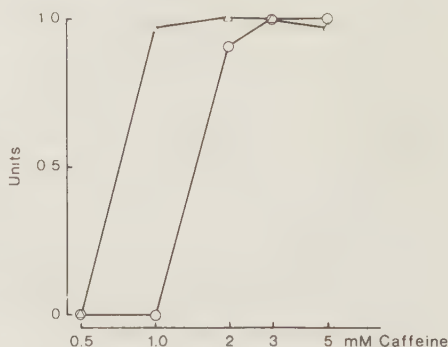


Fig. 2. Effects of DES on peak contracture tension produced by different concentrations of caffeine. O, In the absence of DES. Δ , In the presence of $5 \mu\text{M}$ DES. Each point represents mean value of three different experiments.

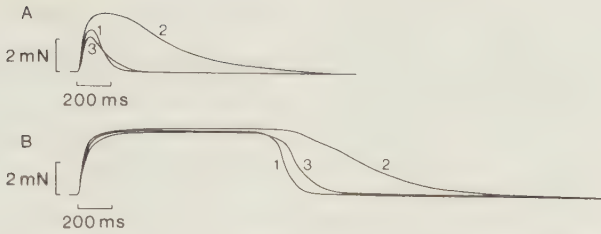


Fig. 3. Effects of dantrolene on twitch (A) and tetanus (B) responses of DES treated fibre. 1. Normal Ringer solution. 2. In the presence of $5 \mu\text{M}$ DES. 3. In the presence of DES and $9 \mu\text{M}$ dantrolene. Stimulus marker below baseline.

the calcium concentration at the myofibrillar sites. If the inhibition by DES is large enough, it would result in an increase in the amplitude of the twitch response and a prolongation of the time to peak twitch tension. Furthermore, as calcium re-sequestration by SR governs muscle relaxation (Hasselbach & Makinose 1961, Ebashi & Lipmann 1962, Weber et al. 1963, Hasselbach 1964, Ebashi & Endo 1968), the twitch potentiation will be accompanied by a prolongation of the relaxation phase. The results presented here are consistent with the idea that DES potentiates the twitch response by inhibiting the SR calcium uptake according to the above mechanism. DES ($100 \mu\text{M}$) inhibits calcium uptake and calcium stimulated ATP-splitting in SR isolated from rabbit skeletal muscle (Khan, unpublished data) which also supports the above mentioned idea.

In mitochondria isolated from human myometrium, DES inhibits the calcium uptake (Batra & Bengtsson 1972). On the other hand, DES exerts an inhibitory action on the contractile activity of the intact uterine smooth muscle and it has been suggested that in this case DES inhibits the influx of calcium through the plasma membrane (Batra & Bengtsson 1978). This idea is consistent with the findings that the contractile activity of the uterine smooth muscle, to a large extent, is dependent on the extra-cellular calcium (Edman & Schild 1962, Bengtsson 1978). It is possible that DES exerts a similar inhibitory action on the plasma membrane of the skeletal muscle, but it is unlikely to produce a noticeable effect, since in this case extra-cellular calcium plays little role in contractile activity (Edman & Grieve 1964, Andersson & Edman 1974, Armstrong et al. 1972 (for review see Fuchs 1974).

DES does not cause any changes in the resting

and action potentials of the fibre. These results suggest that twitch potentiation caused by DES is not due to a membrane effect. DES is a very lipid soluble substance and like other estrogens (Jensen et al. 1969, Evans & Hahnel 1971) may readily penetrate the cell membrane. It is therefore possible that the drug directly affects intracellular structures like the sarcoplasmic reticulum. The membranes of SR have high amounts of phospholipids. Furthermore the SR lipid phase contains large amounts of lecithin (60–73%) (Balzer & Khan 1975, Tada et al. 1978) which have a high percentage of 18-carbon unsaturated fatty acids (Balzer & Khan 1975). Previous studies suggest that drugs like chlorpromazine, reserpine and prenylamine are bound to unsaturated fatty acids of the SR lipid phase and thereby exert their inhibitory action on the SR calcium uptake (Balzer et al. 1968). A similar binding

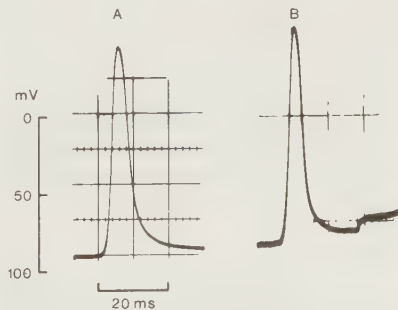


Fig. 4. Effects of DES on intracellularly recorded action potential of frog single muscle fibres. A. Normal Ringer solution. B. In the presence of $5 \mu\text{M}$ DES. The records in A and B are from different fibres.

Table 1. *Effects on resting and action membrane potentials of 5 μ M DES added to normal Ringer (Mean $\pm$ S.E.)*

Frog muscle fibre bundles exposed to DES for 15–20 min before recording the resting and action potentials. Number of fibres for each measurement given within brackets

	Resting potential (mV)	Action potential			
		Overshoot (mV)	Maximum rate of rise (V/s)	Maximum rate of decay (V/s)	Duration at -25 mV level (ms)
Control	88.3 $\pm$ 2.4 (11)	42.9 $\pm$ 2.9 (11)	62.4 $\pm$ 5.1 (11)	31.2 $\pm$ 1.6 (11)	5.2 $\pm$ 0.5 (11)
DES (5 μ M)	87.3 $\pm$ 2.9 (15)	41.9 $\pm$ 4.0 (15)	63.6 $\pm$ 4.0 (15)	29.6 $\pm$ 2.4 (15)	5.0 $\pm$ 0.2 (15)

of DES to the lipid phase of SR might be responsible for the observed twitch potentiation and prolongation of the relaxation phase.

Previously it has been considered that the effects produced by caffeine on excitation-contraction of muscle are due to inhibition of the calcium pump of the SR (Weber 1968). Recent studies of Endo (1975), on the other hand, suggest that caffeine potentiates the twitch and in higher concentration induces contractures by enhancing calcium release from the SR. If DES inhibits the calcium re-uptake by the SR then calcium released by caffeine would accumulate and induce a contracture by lower than normal concentrations of caffeine. The results showing that DES shifted the S-shaped curve relating peak tension and caffeine concentration towards lower drug concentrations are consistent with that idea. Dantrolene, a powerful muscle relaxant, has been suggested to inhibit depolarization-induced calcium release (Hainaut & Desmedt 1974, Desmedt & Hainaut 1977). Inhibition by DES of SR calcium re-sequestration would tend to increase calcium concentration at myofibrillar sites. As this effect is dependent on the first step, i.e. the calcium release, the twitch potentiating effect of DES would be much reduced by dantrolene as was observed in the present experiments.

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Effects of diethyl-stilboestrol on contractile response and action potential of rabbit papillary muscle

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KHAN, A. R. & WOGLFART, B.: Effect of diethyl-stilboestrol on contractile response and action potential of rabbit papillary muscle. *Acta Physiol Scand* 1981, 111: 201–204. Received 30 April 1980. ISSN 0001-6772. Department of Pharmacology, University of Lund, Sweden.

The effects of diethyl-stilboestrol (DES) were studied on isolated papillary muscles from rabbits (37°C). The preparation was paced at a frequency of 0.67 Hz and the muscle length was adjusted to 95% of the length of optimum force production. Membrane potentials and the isometric force production were recorded. DES (10 μ M) reduced peak isometric force by 44.5% (mean of 6 preparations) and maximum rate of force development was reduced to approximately the same degree. The time to peak force, the time from peak force to half relaxation and the action potential duration were not significantly affected by DES. The results are consistent with the idea that DES reduces the calcium influx during the cardiac action potential without significantly influencing the calcium reuptake during relaxation. The data are compared to the effects of DES on uterine and skeletal muscles and the differences in metabolism of activator calcium in different kinds of muscles are discussed.

Key words: Diethyl-stilboestrol, myocardium, sarcoplasmic reticulum, excitation-contraction coupling, calcium metabolism

It has previously been shown that diethyl-stilboestrol (DES) significantly influences the metabolism of activator calcium during the excitation-contraction process in both skeletal and uterine muscle. Batra & Bengtsson (1978) suggested that the relaxing effect of DES on uterine muscle is due to inhibition of calcium influx through the plasma membrane. This assumption is in line with the idea that contractile activity of uterine smooth muscle depends, to a large extent, on calcium influx from the extracellular space (Bengtsson 1978). Transmembrane calcium fluxes, most likely, play no predominant role in contractile activity of skeletal muscle (for review see Ebashi 1976). DES has been reported to potentiate the amplitude of the twitch response and also to prolong the relaxation phase of the contraction of single muscle fibres (Khan 1979). It seems reasonable that these effects of DES are due to inhibition of the reuptake of calcium by the sarcoplasmic reticulum during relaxation.

Contractile activation of cardiac muscle is believed to be dependent on both transsarcolemmal calcium fluxes and intracellular movements of cal-

cium (for reviews see Fozzard 1977 and Fabiato & Fabiato 1979). It was therefore of interest to investigate in somewhat detail the effects of DES on the contractile response of myocardium. Preliminary results of this study have been reported at the Scandinavian Physiological Society Meeting (Khan & Wohlfart 1980).

METHODS

Preparation and mounting. Papillary muscles (2–4 mm in length and 0.4–0.8 mm in diameter) were dissected from the right ventricle of small (about 1 kg weight) rabbits. The preparation was mounted horizontally, in a thermostatically controlled bath (36.5–37.5°C), between a force transducer and a steel hook. The muscle was stimulated to contract isometrically by passing current from a Grass S-44 stimulator between two electrodes (made from platinum wire) placed at either side of the muscle. The stimulus pulse was a unidirectional rectangular wave of 2 ms duration and with an amplitude about 50% above the threshold value. The muscle was paced to contract at a rate of 0.67 Hz. The length of the muscle was adjusted to 95% of the length of optimum force production.

Recordings of mechanical and electrical responses. Isometric force was recorded with a strain-gauge trans-

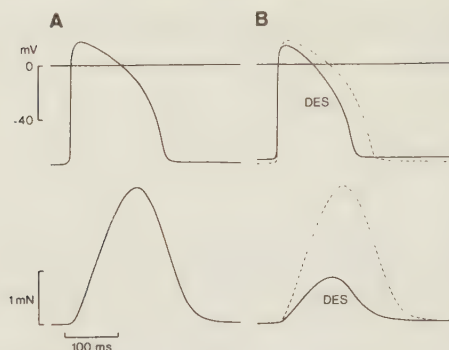


Fig. 1. The effect of DES on action potential (upper panels) and isometric force production (lower panels) of an isolated papillary muscle (paced at 0.67 Hz, temperature 37°C). Recordings are before (in panel A) and after (in panel B) exposure to 10 μ M DES. Controls from panel A are redrawn (interrupted lines) in panel B for comparison.

ducer that was connected to one end of the muscle through a glass hook. Conventional glass capillary electrodes filled with 2.5 M KCl and with a resistance of 10–30 Mohm were used to record the membrane potential. Action potentials and myograms were recorded by a jet ink-writer (Elema-Schönander mingograf) at a low paper speed. Selected recordings were displayed on a Tektronix 5103N storage oscilloscope and photographed on 35 mm orthochromatic film.

Film analysis. Measurements of the oscilloscope records were made at 10 $\times$ magnification in a Nikon model 60 profile projector. The stage micrometer readings were used to calculate the amplitude of the myogram. The maximum slope of the force production was measured with the Nikon vernier scale. Time to peak force was taken as the time from the stimulus to the peak of the

contraction. Time from peak force to half relaxation was also measured. The action potential duration was defined as the time from the stimulus to the repolarization of the action potential at a voltage of -40 mV.

Solutions. The muscle preparation was continuously perfused (flow rate about 4 ml/min) with oxygenated (95% O₂ and 5% CO₂) solution of the following composition (mM): NaCl 100, KCl 4, MgSO₄ 1.5, CaCl₂ 2.0, NaHCO₃ 20, glucose 10 and Na-acetate 20. The solution also contained insulin (2U/l). The pH of the solution was 7.4. All chemicals used were of analytical grade and dissolved in double-distilled water. Diethylstilboestrol (DES) was obtained from Sigma Chemical Co., St. Louis, USA. DES was dissolved in absolute alcohol from which bathing solutions containing 1–15 μ M DES were prepared. Maximum alcohol concentration did not exceed 1.0 ml/l solution. No significant effect of this amount of alcohol was observed on contractile force.

RESULTS

The effects of diethylstilboestrol (DES) on the isometric contraction were studied in isolated papillary muscles. Exposure of the preparation to 1 μ M DES for 30 min did not significantly affect the force production. DES, in a higher concentration, reduced peak isometric force and this effect was seen approximately 10 min after the introduction of DES. Fig. 1 shows the effect of DES (10 μ M) on the time course of the isometric twitch (lower traces). It can be seen that DES greatly reduced peak force and also the rate of force development (Panel B). Fig. 1 also shows the simultaneously recorded action potential (upper traces). It is seen in panel B that DES reduced the duration of the action potential. The overshoot of the action potential was also reduced by DES.

Table 1. Effects of DES on cardiac action potential and isometric force production

Values are given as percentage change of control values in each preparation after exposure to 10 μ M DES. Mean of controls were: 160 ms (action potential duration), 6.6 mN/mm² (peak force), 78.0 mN/mm²·s (max. rate of force development), 133 ms (time to peak force) and 60 ms (time from peak force to half relaxation)

Experiment number	Action potential duration	Peak force (F)	Maximal dF/dt	Time to peak force	Time to half relaxation
1	+ 4.4	-40.0	-34.9	$\pm$ 0.0	-13.2
2	- 5.8	-27.1	-30.4	+ 2.9	+ 1.7
3	- 1.9	-66.7	-62.1	-10.8	- 5.5
4	- 3.8	-67.1	-59.8	-13.5	+ 5.6
5	-11.3	-46.7	-43.1	- 8.5	- 8.6
6	+ 3.6	-19.4	-18.1	- 3.3	- 7.0
Mean change	- 2.5	-44.5*	-41.4*	- 3.5	- 4.5
$\pm$ S.E.	$\pm$ 2.4	$\pm$ 8.1	$\pm$ 7.0	$\pm$ 2.6	$\pm$ 2.8

* Significant effect, $P < 0.01$

Table 1 summarizes the effect of about 30 min exposure to 10 μ M DES on cardiac action potential and isometric force production in six different preparations. The percentage change of the variables are given for each preparation. The table demonstrates that DES shortened the action potential in 4 out of 6 muscles. The mean change of the duration, however, was not significantly affected. Peak isometric force and maximum rate of force production were significantly reduced by DES; the percentage reduction was about the same for both. The data also shows that time to peak force and time from the peak to half relaxation was not significantly affected by DES.

The negative inotropic effect of DES often continued to develop even after 30 min exposure to the drug and the effect could not be fully reversed by reperfusion with control solution.

DISCUSSION

Diethyl-stilboestrol (DES) reduced peak isometric force and also maximum rate of force development of the isolated papillary muscle. It has been shown previously that DES inhibits the contraction of isolated uterine muscle (Hempel & Neumann 1965, Saldívar & Melton 1966, Mossman & Conrad 1967, Bengtsson 1978) and Batra & Bengtsson (1978) provided evidence suggesting that this effect was due to inhibition of calcium influx across the cell membrane. It appears likely that the negative inotropic effect observed in cardiac muscle is also due to a reduced calcium influx during excitation. This is in keeping with the idea that the contractile state of uterine muscle (Edman & Schild 1962, 1963), as well as that of cardiac muscle (for a review see Fozzard 1977), is critically dependent on the extracellular calcium pool. In skeletal muscle, on the other hand, calcium movements during the excitation-contraction cycle are generally believed to form a virtually closed system with a relatively small exchange of calcium across the cell membrane. On this basis DES would not be expected to depress the contractile response of skeletal muscle. In fact, as demonstrated in a previous study (Khan 1979), DES exerts a potentiating effect on the twitch response of isolated muscle fibres. This effect is associated with a pronounced slowing of the relaxation phase of both twitch and tetanus. These findings suggest that DES inhibits the rate of

calcium resequestration by the sarcoplasmic reticulum (SR) during relaxation.

It is noteworthy that DES did not affect the rate of relaxation of papillary muscle. This could mean that the SR in myocardium is less sensitive to DES than is the SR in skeletal muscle. Alternatively, the SR may play a minor part in the relaxation of heart muscle. It should be noted that the volume of SR is considerably smaller in cardiac than in skeletal muscle. The ratio of the SR volume to the total cell volume is approximately 0.3 for cardiac muscle as compared to 4.1 for skeletal muscle (Fabiato & Fabiato 1977). As proposed by Lüllmann & Peters (1977), Seibel et al. (1978), and others, relaxation of myocardium may largely be accomplished by the binding function of the sarcolemma.

The effect of DES on the duration of the cardiac action potential was variable. In 4 expts a shortening of the action potential was seen whereas in two experiments the action potential was prolonged. This variability may be explained by assuming a dual effect of DES. The direct effect of DES on the cell membrane is assumed to be to shorten the action potential and to reduce the intensity of the calcium influx (cf above). However, the ensuing negative inotropic effect (due to a smaller amount of calcium in the cellular store) will tend to prolong the action potential because of a feedback coupling between inotropic state and action potential duration (Braveny & Sumner 1970, Wohlfart 1979 and Jóhannsson & Wohlfart 1980). It is of interest to note that the same feedback mechanism operates when the extracellular calcium is reduced. This intervention leads to a reduced calcium uptake and a smaller force production which is accompanied by a prolonged action potential (Bass 1975).

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Mechanism of action of pentobarbital on the contractile system of isolated frog muscle fibres

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The effects of pentobarbital-sodium were studied on single muscle fibres of frog skeletal muscle. In a concentration of 0.5 mM, pentobarbital potentiated the twitch response without significantly affecting the tetanus amplitude. The increase in twitch amplitude was accompanied by a marked increase in the rate of force development. The relaxation phase of both twitch and tetanus was prolonged. These mechanical changes were associated with an increased duration of the action potential. Pentobarbital also increased the amplitude and the rate of force development during the twitch after suppression by dantrolene. The S-shaped curve relating peak contracture tension and log caffeine concentration was shifted to the left by pentobarbital. It is suggested that pentobarbital affects the contractile activity of the muscle fibre; (1) by increasing the rate of release of calcium from its storage sites, (2) by prolonging the duration of the action potential and (3) by inhibiting calcium resequestration by the sarcoplasmic reticulum (SR).

Key words: Pentobarbital, skeletal muscle, single muscle fibre, excitation-contraction coupling, twitch potentiation, action potential

Barbiturates are known to have direct effect on muscle contraction. Pentobarbital, for example, has been shown to potentiate the twitch response of mammalian as well as amphibian muscles (Quilliam 1955*a*, Gjone 1956, Foulks et al. 1973, Holmberg & Waldeck 1979). The stimulatory effect of barbiturates on the twitch response of muscle has been attributed to an increased duration of the action potential (Quilliam 1955*b*, Gjone 1956, Foulks et al. 1973), although it has been suggested that they may augment the twitch by interfering with the relaxation mechanism (Gjone 1956). This latter assumption is supported by the findings that barbiturates inhibit calcium uptake by the sarcoplasmic reticulum (SR) isolated from heart (Briggs et al. 1966).

The present study was undertaken as an attempt to obtain further information concerning the mode of action of barbiturates on skeletal muscle. Evidence will be presented to show that pentobarbital greatly potentiates the twitch response and also prolongs relaxation in isolated frog muscle fibres. The twitch potentiation may be attributed to an increased duration of the action potential. It is

suggested that the slowing of the relaxation is due to an inhibitory effect of pentobarbital on calcium re-sequestration by the SR.

METHODS

Effects of pentobarbital were studied on muscle fibres isolated from the ventral head of the semitendinosus muscle of *Rana temporaria*. The methods used for mounting, stimulation, temperature control and for production of caffeine contracture are described elsewhere (Khan & Edman 1979).

Recording of membrane potential. Resting and action membrane potentials were recorded in the presence and absence of pentobarbital (0.5 mM) using conventional glass capillary electrodes (Khan & Edman 1979).

In these experiments the bath temperature varied between 2.8 and 3.3°C but was maintained constant to within $\pm 0.2^\circ\text{C}$ throughout any particular experiment, even during exchange of solutions.

Solutions. A Ringer solution of the following composition was used (mM): NaCl 115.5, KCl 2.0, CaCl_2 1.8, Na-phosphate buffer 2.0 pH 7.0.

Pentobarbital-sodium (0.05–1.0 mM), barbitone-sodium (1.0 mM) and dantrolene-sodium (9.0 μM) were dissolved in Ringer solution and care was taken to adjust the pH to 7.00–7.15.

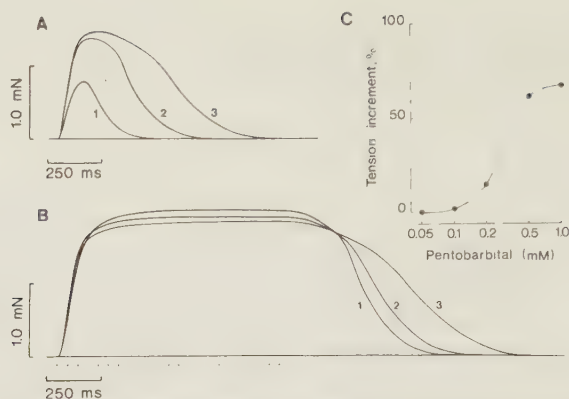


Fig. 1. Effects of pentobarbital-sodium on isometric twitch (A) and tetanus (B) in frog single muscle fibre. (1) Control, normal Ringer solution. (2) 0.5 mM pentobarbital. (3) 1.0 mM pentobarbital. Stimulus markers below the base line. Concentration-response curve (C). Ordinate, increase in twitch tension in per cent of control (no pentobarbital). Abscissa, pentobarbital concentration (log scale). Each point represents mean value of 3 expts.

All chemicals used were of analytical grade and were dissolved in double distilled water.

Pentobarbital-sodium and barbitone-sodium were obtained from B.D.H. Chemicals, Poole, England. Dantrolene-sodium was from Eaton Chemicals, Norwich, N.Y., USA.

RESULTS

The effects of pentobarbital were studied on twitch and tetanus responses of single muscle fibres. It can be seen in Fig. 1A that pentobarbital (0.5 and 1.0 mM) greatly potentiated the twitch amplitude and increased the rate of development of tension. According to the concentration-response curve (Fig.

1C), maximum potentiation of the twitch was obtained with 1.0 mM pentobarbital. The increased amplitude of the twitch was accompanied by a slight increase in the time to peak tension and a marked prolongation of the relaxation phase (Fig. 1A and Table 1). The magnitude of the twitch potentiation was inversely related to the twitch/tetanus ratio recorded in normal Ringer solution. When the effect of pentobarbital was maximal (1.0 mM), the amplitude of the twitch reached about 90% of the tetanic force.

Pentobarbital (0.5 mM) caused no significant change in the amplitude of the tetanus response (Fig. 1B). At a bath concentration of 1.0 mM pento-

Table 1. Effects of pentobarbital (0.5 mM) on the twitch parameters in frog single muscle fibres

Each value represents the mean of 4-6 twitch responses

Expt. no.	Normal Ringer		0.5 mM pentobarbital		T_p/T_c	R_p/R_c
	Time for 1/2 peak tension (ms) T_c	Time for 1/2 relaxation (ms) R_c	Time for 1/2 peak tension (ms) T_p	Time for 1/2 relaxation (ms) R_p		
1	61	96	79	219	1.3	2.3
2	53	114	70	324	1.3	2.8
3	61	88	88	167	1.4	1.9
Grand mean	58	99	79	236	1.3	2.3

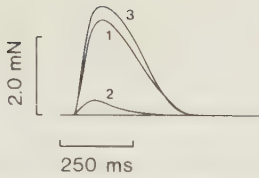


Fig. 2. Effects of pentobarbital on isometric twitch of frog single muscle fibre pretreated with dantrolene. (1) Control, normal Ringer solution. (2) In the presence of dantrolene ($9.0 \mu\text{M}$). (3) In the presence of dantrolene ($9.0 \mu\text{M}$) and pentobarbital (0.2 mM).

barbital, the tetanic force, however, was slightly depressed. Similar to the situation in the twitch response, the time to half decay of tension after the last stimulus of the tetanus response was also prolonged.

The effects produced by pentobarbital were, to a large extent, reversible by repeated washing of the fibre with Ringer solution. An increase of the pentobarbital to 2.0 mM rendered the fibre inexcitable to electrical stimulation.

Dantrolene, a powerful muscle relaxant, is known to suppress the twitch response in a single muscle fibre (Hainaut & Desmedt 1974, Desmedt & Hainaut 1977, Edman 1979). The effects of pentobarbital were also studied in the presence of dantrolene ($9.0 \mu\text{M}$). As illustrated in Fig. 2, pentobarbital (0.2 mM) potentiated the dantrolene suppressed twitch with a marked increase in the rate of force development.

Contractures were induced by different concentrations of caffeine in the presence and absence of pentobarbital (0.5 mM). The peak tension developed was plotted against the log concentration of caffeine. As illustrated in Fig. 3, pentobarbital shifted the S-shaped curve relating contracture tension and log caffeine concentration to the left.

Other barbiturates like barbitone, with low lipid solubility, seem to have little effect on the contractile activity of the muscle fibre. As illustrated in Fig. 4, barbitone in a concentration of 1.0 mM had almost no effect on the twitch response.

Influence of pentobarbital on resting and action membrane potential

Pentobarbital did not cause any significant change of the resting membrane potential. The overshoot and the maximum rate of rise of the action potential were also not changed significantly. Pentobarbital,

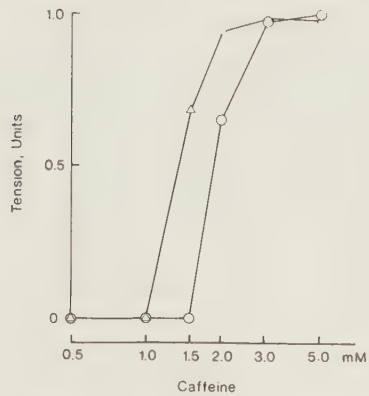


Fig. 3. Effects of pentobarbital on the relation between peak contracture tension and caffeine concentration (log scale) in a single muscle fibre. ○, in the absence of pentobarbital. Δ, in the presence of pentobarbital (0.5 mM). The maximum tension developed by 5 mM caffeine in the absence of pentobarbital was taken as 1.0 unit. Each point represents mean value of 3 different expts.

however, reduced the rate of repolarization, causing an increased duration of the action potential (Fig. 5 and Table 2). These results are consistent with earlier findings in studies on whole muscle preparations (Quilliam 1955b, Thesleff 1956, Foulks et al. 1973).

DISCUSSION

The results presented here show that pentobarbital potentiates the isometric twitch amplitude of the single muscle fibre and increases the duration of the action potential. These findings are in agreement with results obtained previously in studies of mammalian and amphibian whole muscles (Quilliam

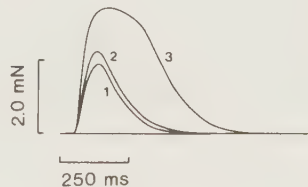


Fig. 4. Comparison of pentobarbital and barbitone effects on isometric twitch of single muscle fibre. (1) Control, normal Ringer solution. (2) In the presence of 1.0 mM barbitone. (3) In the presence of 0.5 mM pentobarbital.

Table 2. Effects of pentobarbital (0.5 mM) on resting and action membrane potentials of frog muscle fibres (mean $\pm$ S.E.)

Before recordings were made, the fibre bundles were exposed to pentobarbital for 15–20 min. Number of fibres for each measurement given within brackets. Student's *t*-test; *** $P < 0.001$

	Resting potential (mV)	Action potential			
		Overshoot (mV)	Maximum rate of rise (V/s)	Maximum rate of decay (V/s)	Duration at -25 mV level (ms)
Control	86.9 $\pm$ 2.7 (12)	40.0 $\pm$ 2.8 (12)	64.2 $\pm$ 5.1 (12)	27.7 $\pm$ 1.0 (12)	4.8 $\pm$ 0.2 (12)
Pentobarbital	84.4 $\pm$ 2.3 (12)	37.4 $\pm$ 2.9 (12)	63.2 $\pm$ 6.4 (12)	18.5 $\pm$ 1.5 (12)	6.2 $\pm$ 0.4 (12)

1955*b*, Gjone 1956, Thesleff 1956, Foulks et al. 1973). As the action potential may be assumed to govern the release of activator calcium (Sandow et al. 1965, Edman et al. 1966, Taylor et al. 1972) its prolongation would cause an increased release of calcium from the storage sites. However, if this were the only factor causing twitch potentiation then there would be no change of the initial rate of rise of tension. The fact that the rate of force development is markedly steeper in the presence of pentobarbital, suggests that the rate of calcium release is increased, not merely the duration of the release. Further evidence in support of this idea is provided by the fact that pentobarbital counteracts the effects of dantrolene. The latter agent inhibits the depolarization-induced release of calcium from the storage sites (Hainaut & Desmedt 1974, Desmedt & Hainaut 1977) leading to a marked decrease of both the rate of rise and amplitude of the twitch (Hainaut & Desmedt 1974, Desmedt &

Hainaut 1977, Edman 1979). Pentobarbital restored the speed of force development, in the presence of dantrolene suggesting that the rate of release of calcium was indeed increased.

On the basis of studies carried out on muscle from the rat diaphragm, Gjone (1956) suggested that barbiturates affect muscle contraction by inhibiting the relaxation process. It is now well established that relaxation in skeletal muscle is achieved when calcium is resequestered by the sarcoplasmic reticulum (SR). An inhibitory action of pentobarbital on the calcium pump would, therefore, prolong the relaxation phase of the twitch as well as of the tetanus response as was actually observed in the present experiments.

If pentobarbital inhibits the reuptake of calcium by SR, this would tend to raise the calcium concentration in the myofibrillar space during calcium release by caffeine. As caffeine is assumed to induce contractures by enhancing the release of calcium from the storage sites (Endo 1975), this would be expected to lower the concentration of caffeine required to induce contracture. In accordance with this prediction, pentobarbital was found to shift the S-shaped curve relating peak contracture tension and log caffeine concentration to the left. This suggests that pentobarbital, like diethylstilboestrol, may have an inhibitory action on the SR calcium resequestration. Such an effect on calcium pump of the SR may also contribute to the twitch potentiation as suggested previously (Khan 1979).

It appears likely that pentobarbital, being very lipid soluble, readily penetrates the cell membrane to have access to intracellular structures like SR. The SR membranes are rich in phospholipid contents and have a high percentage of phosphatidyl-

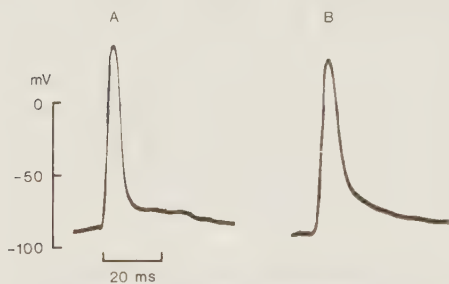


Fig. 5. Intracellular recordings of action potentials. (A) In normal Ringer solution. (B) In the presence of 0.5 mM pentobarbital. The recordings in A and B are from different muscle fibres and slightly retouched for greater clarity.

choline (60–73%) (Balzer & Khan 1975, Tada et al. 1978). Previous studies suggest that drugs like reserpine, chlorpromazine and prenlyamine exert their inhibitory action on SR calcium uptake by binding to the unsaturated fatty acids of the SR lipid phase (Balzer et al. 1968). A similar binding of pentobarbital to the lipid phase of the SR might cause a slowing of calcium reuptake and, hence, be responsible for the prolongation of the relaxation phase.

In conclusion pentobarbital seems to influence the contractile activity of the muscle fibre in 3 different ways:

1. By enhancing the *rate* of release of calcium from the storage sites, pentobarbital causes an increased rate of force development and an increased amplitude of the isometric twitch.

2. By prolonging the duration of the action potential the release of calcium occurs over a longer time. This effect will add to factor 1 causing an increased amplitude of the isometric twitch.

3. Pentobarbital probably inhibits the calcium re-sequestration by the SR leading to a reduced rate of relaxation of the twitch and tetanus response.

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Influence of ethanol and acetaldehyde on electro-mechanical coupling of skeletal muscle fibres

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KHAN, A. R.: Influence of ethanol and acetaldehyde on electro-mechanical coupling of skeletal muscle fibre. *Acta Physiol Scand* 1981. 111: 425-430. Received 4 July 1980. ISSN 0001-6772. Department of Pharmacology, University of Lund, Sweden.

The effects of ethanol were studied on the time course of the isometric twitch and tetanus of isolated semitendinosus muscle fibres of *Rana temporaria* (at 2.8-4.5°C). Ethanol in concentrations between (0.1-0.4 M) suppressed the isometric twitch without significantly affecting the tetanus amplitude. A further increase in ethanol concentration (0.5 M) depressed the tetanic tension as well. The maximum rate of force development both of twitch and tetanus was reduced. These changes were fully reversible when ethanol was removed from the bathing fluid. In presence of ethanol there was a reduction, although not statistically significant, in the resting membrane potential and in the maximum rate of rise of the action potential. The overshoot and the maximum rate of fall of the action potential were significantly lowered leading to a prolongation of the duration of the action potential. Acetaldehyde in relatively low concentrations (0.9-1.8 mM) caused a marked potentiation of the twitch without significantly affecting the tetanic amplitude. These changes were, to a great extent, reversible. Higher concentrations (18 mM) of acetaldehyde reduced both the twitch and the tetanic tension and these effects were not reversible on removal of drug from the bathing solution. No detectable effects of the drug (1.8 mM) were observed on resting or action membrane potentials of the muscle fibre. The data presented supports the idea that ethanol suppresses the twitch response by inhibiting the calcium release mechanism and uncouples the excitation-contraction process. The twitch potentiation by acetaldehyde seems to be due to enhancement of calcium release from the storage sites.

Key words: Ethanol, acetaldehyde, skeletal muscle, single muscle fibre, excitation contraction coupling, action potential, membrane potential

Chronic ingestion of ethanol has been reported to cause damage to the human muscle which is accompanied by striking ultrastructural changes in the muscle cell (Song & Rubin 1972). Ethanol effects on muscle were studied by Gage (1965) using curarized frog sartorius muscle. He reported that the directly stimulated muscle contractility was greatly reduced by ethanol. In a muscle fibre ethanol also lowered the resting membrane potential (Knutsson 1961). However, little is known about the action of acetaldehyde, the first metabolite of ethanol, on skeletal muscle. The present studies were, therefore, undertaken to investigate the effects of ethanol and acetaldehyde on excitation-contraction coupling using isolated muscle fibres.

Evidence will be presented showing that ethanol

and acetaldehyde, in high concentrations, affect the twitch responses. Ethanol markedly suppresses the rate of force development both in twitch and tetanus responses suggesting an inhibitory action of the drug on calcium release mechanisms. On the contrary acetaldehyde greatly potentiates the twitch probably by enhancing the calcium release from the intracellular storage sites.

METHODS

Single muscle fibres dissected from the ventral head of the semitendinosus muscle of *Rana temporaria* were used. The apparatus and the techniques used for recording of twitch and tetanus and caffeine contractures and for the recording of resting and action membrane potentials were the same as described previously (Khan & Edman 1979).

Table 1. Effects on resting and action membrane potentials of 0.4 M ethanol added to normal Ringer (mean $\pm$ S.E.)

Temperature (2.8–4.5°C). Frog muscle fibre bundles exposed to ethanol for 15–20 min before recording the resting and action potentials. Number of fibres for each measurement given in brackets. *Significant effect, $P < 0.01$

	Resting potential (mV)	Action potential			
		Overshoot (mV)	Maximum rate of rise (V/s)	Maximum rate of decay (V/s)	Duration at -25 mV level (ms)
Control	86.7 $\pm$ 1.6 (11)	37.2 $\pm$ 2.7 (11)	57.3 $\pm$ 2.7 (11)	17.2 $\pm$ 0.5 (11)	6.9 $\pm$ 0.2 (11)
Ethanol (0.4 M)	82.7 $\pm$ 1.5 (11)	25.3* $\pm$ 2.8 (11)	46.0 $\pm$ 4.4 (11)	8.7* $\pm$ 1.1 (11)	9.2* $\pm$ 0.4 (11)

In these expts. the bath temperature varied between 2.8–4.5°C and was maintained constant to within $\pm 0.2^\circ\text{C}$ throughout any particular expt., even during exchange of solutions.

Solutions containing 0.05–0.5 M ethanol and 0.9–1.8 mM acetaldehyde were prepared by dissolving the respective drugs in the Ringer solution. No correction was made for osmolality when the drugs were used. A 0.9 μM solution of dantrolene sodium was prepared in Ringer solution.

All chemicals used were of analytical grade and dissolved in double distilled water. Dantrolene sodium was from Eaton Chemicals, Norwich, N.Y., USA, and acetaldehyde was obtained from BDH, Poole, England.

RESULTS

As illustrated in Fig. 1 A, ethanol (0.4 M) markedly suppressed the twitch response without significantly affecting the tetanus amplitude (Fig. 1 B). The maximum rate of force development was

reduced during both twitch and tetanus (Fig. 1 A and B). A depression of tetanic amplitude was noticed when higher concentrations (0.5 M) of ethanol were used (Fig. 2 A and B). The contractile effects produced by ethanol (0.4–0.5 M) were fully reversible on removal of ethanol from the bathing fluid (Figs. 1 and 2, A and B).

Resting and action membrane potentials were also recorded after 15–20 min exposure to ethanol (0.4 M). It can be seen from Table 1 that there was a reduction, although not statistically significant, of the resting membrane potential and the maximum rate of rise of the action potential. However, the overshoot and the maximum rate of fall of the action potential were significantly reduced leading to an increase in duration of the action potential (Table 1).

No detectable change in fibre width was noticed in the presence of 0.4–0.5 M ethanol as measured

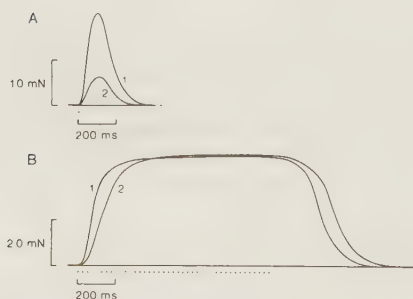


Fig. 1. Effects of 0.4 M ethanol on isometric twitch (A) and tetanus (B) responses. 1. Normal Ringer, identical responses obtained before and after ethanol treatment. 2. Ethanol (0.4 M). Note different tension scales in A and B. Stimulus markers below base line.

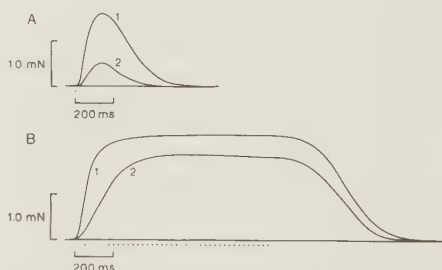


Fig. 2. Effects of 0.5 M ethanol on isometric twitch (A) and tetanus (B) responses. 1. Normal Ringer, identical responses obtained before and after ethanol treatment. 2. Ethanol (0.5 M).

Table 2. Effects on resting and action membrane potentials of 1.8 mM acetaldehyde added to normal Ringer (mean $\pm$ S.E.)

Temperature (2.8–4.5°C). Frog muscle fibre bundles were exposed to acetaldehyde for 15–20 min before recording the resting and action potentials. Number of fibres for each measurement given in brackets

	Resting potential (mV)	Action potential			
		Overshoot (mV)	Maximum rate of rise (V/s)	Maximum rate of decay (V/s)	Duration at -25 mV level (ms)
Control	85.0 $\pm$ 0.9 (15)	39.1 $\pm$ 1.2 (15)	57.0 $\pm$ 2.3 (15)	16.4 $\pm$ 0.8 (15)	7.7 $\pm$ 0.3 (15)
Acetaldehyde (1.8 mM)	85.9 $\pm$ 0.9 (14)	37.2 $\pm$ 2.0 (14)	58.6 $\pm$ 3.8 (14)	17.1 $\pm$ 1.0 (14)	7.3 $\pm$ 0.2 (14)

by an ocular micrometer at 40 $\times$ magnification.

Acetaldehyde in relatively low concentrations, ranging from 0.9 to 1.8 mM, had a marked potentiating effect on the isometric twitch (Fig. 3 A) without affecting the tetanic amplitude (Fig. 3 B). There was no significant change in the initial rate of rise of tension (Fig. 3 A and B). These changes were, almost completely, reversible when acetaldehyde was removed from the bathing solution (Fig. 3 A and B). The higher concentrations (18 mM) of acetaldehyde depressed both the twitch and the tetanus (Fig. 4 A and B) and in this case the effects were only partially reversible after the removal of the drug.

Acetaldehyde in very high concentration (0.9–1.8 M) induced a sustained contracture. Relaxation could be produced by repeated washing with nor-

mal Ringer but the twitch response was not restored after such a contracture.

Acetaldehyde in twitch potentiating concentration (0.9–1.8 mM) did not affect the contractures induced by caffeine (1–3 mM).

The resting and the action membrane potentials were recorded after 15–20 min exposure to acetaldehyde (1.8 mM). It can be seen from Table 2 that acetaldehyde caused no significant change in the resting and action membrane potentials.

Ethanol greatly reduced the contractile activity of the muscle fibre. The twitch potentiating effect of acetaldehyde was, therefore, tested in presence of ethanol (0.4 M). As illustrated in Fig. 5 A, acetaldehyde, to a great extent, counteracted the ethanol effects.

Similarly, dantrolene, a powerful muscle relax-

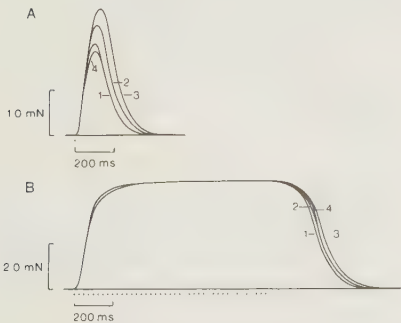


Fig. 3. Effects of acetaldehyde on isometric twitch (A) and tetanus (B) responses. 1. Normal Ringer. 2. 0.9 mM acetaldehyde. 3. 1.8 mM acetaldehyde. 4. Normal Ringer, after acetaldehyde treatment.

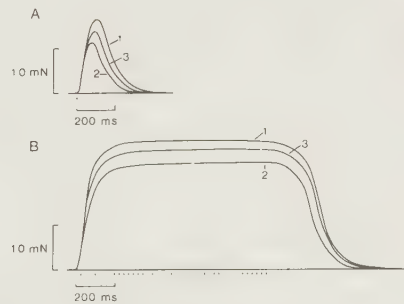


Fig. 4. Effects of 18 mM acetaldehyde on isometric twitch (A) and tetanus (B) responses. 1. Normal Ringer. 2. 18 mM acetaldehyde. 3. Normal Ringer, after acetaldehyde treatment.

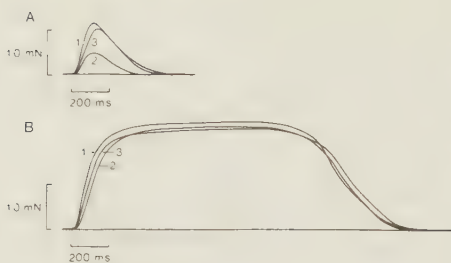


Fig. 5. Combined effects of ethanol and acetaldehyde on isometric twitch (A) and tetanus (B) responses. 1. Normal Ringer. 2. 0.4 M ethanol. 3. 0.4 M ethanol and 1.8 mM acetaldehyde.

ant, is known to depress the twitch response of a muscle fibre in micromole concentrations (Desmedt & Hainaut 1977, Hainaut & Desmedt 1974, Khan & Edman 1979, Edman 1979). When acetaldehyde (1.8 mM) was tested on dantrolene (9.0 μ M) treated fibres, acetaldehyde, to some extent, reversed the dantrolene effects as well (Fig. 6 A and B).

DISCUSSION

The present results show that ethanol in high concentration (0.4 M) greatly suppress the contractile activity of the muscle fibre which is in good agreement with results obtained on frog whole skeletal muscle (Gage 1965) and rat atrial muscle (Gimeno et al. 1962). No potentiating effect of ethanol was observed although as low as 0.01 M concentrations were tested. The previously reported contractile potentiating effect of low ethanol concentrations on indirectly stimulated nerve-muscle preparations (Ettinger et al. 1941, Sachdev 1963) may, therefore, be due entirely to enhancement of the neuromuscular transmission as suggested by Gage (1965).

Previous work suggests that there exists a direct relationship between the duration of the action potential and the duration of the mechanical activity. The prolongation of the action potential may be assumed to increase the time during which activator calcium is released into the myofibrillar space and this is reflected by an increase of the twitch amplitude (Sandow et al. 1964, Sandow & Preise 1964, Sandow et al. 1965, Edman et al. 1966, Taylor et al. 1972, Andersson & Edman 1974, Khan & Edman 1979, Khan 1980). Ethanol significantly prolongs the duration of the action potential and thus

a potentiation of the twitch would be expected. However, ethanol depresses the twitch amplitude which suggests that the drug may have dual effect. It prolongs the time during which activator calcium is released and has a strong inhibitory action on the calcium release mechanism. The latter view is further supported by the observation that the rate of tension development in the twitch and tetanus responses is also very much reduced.

These results suggest that ethanol, in high concentration, uncouples the excitation-contraction mechanism. The possibilities cannot be excluded, however, that ethanol may decrease the sensitivity of the contractile system to activator calcium.

A depolarizing effect of ethanol on the frog muscle fibre has been reported previously and this effect was observed at room temperature (22–25°C) and in an atmosphere of pure oxygen (Knutsson 1961). No significant decrease in resting membrane potential was noticed which may be due to the fact that the presented experiments were carried out at a low temperature (2.8–4.5°C). The possibilities were considered that ethanol produces an osmotic effect and thus would be expected to reduce the mechanical activity (Edman & Hwang 1977). It seems unlikely, because no detectable change in the muscle fibre width was noticed in presence of ethanol. This is in accordance with the earlier view that small ethanol molecule rapidly equilibrates across the fibre membrane and therefore has little osmotic effect (Abel 1980).

By contrast to ethanol acetaldehyde markedly potentiates the twitch response. In concentrations having maximum twitch potentiation, acetaldehyde neither affects the resting potential nor the action potential. Like 4-aminopyridine (Khan & Edman

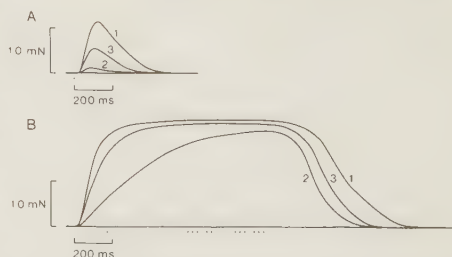


Fig. 6. Effects of acetaldehyde in the presence of dantrolene on isometric twitch (A) and tetanus (B) responses. 1. Normal Ringer. 2. 9.0 μ M dantrolene. 3. 9.0 μ M dantrolene and 1.8 mM acetaldehyde.

1979), acetaldehyde potentiates the twitch response even in the absence of calcium in the extra-cellular medium. This is in line with the idea that acetaldehyde causes no increase in trans-membrane calcium influx. It is, therefore, assumed that twitch potentiation by acetaldehyde is due to a more direct effect on the intracellular calcium metabolism. In very high concentrations acetaldehyde induced contractures in the muscle fibre. This observation supports the idea that acetaldehyde, like caffeine (1) potentiates the twitch by enhancing the release of calcium and (2) induces contractures by initiating the calcium release from the intracellular storage sites. However it differs from caffeine in the sense that there is a very big difference between its twitch potentiating and contracture inducing concentrations.

It would be expected that acetaldehyde may exert synergic action on caffeine induced contractures in muscle fibre and thus lower the concentration of caffeine required to induce contracture normally. In twitch potentiating concentrations no such effect of acetaldehyde was noticed in these experiments. It should be, however, recalled that caffeine contractures, very frequently, show all-or-none response (Endo 1975) and therefore a small decrease of the concentration of caffeine necessary for inducing contracture in presence of acetaldehyde may be hard to notice.

There is now ample evidence suggesting that dantrolene suppress the twitch response of the muscle fibre by inhibiting calcium release from the storage sites (Putney & Bianchi 1974, Van Winkle 1976, Desmedt & Hainaut 1977, Morgan & Bryant 1977). Acetaldehyde to some extent reverses this effect, probably by counteracting the inhibitory action of dantrolene on calcium release mechanism.

A similar countereffect by acetaldehyde seems to be involved in reversing the ethanol suppressed twitch response.

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